RNA AND PROTEIN SYNTHESIS
IN THE DIFFERENTIATION OF THE LENS

by

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SUMMARY

The aim of this study was to gain an insight into gene expression during differentiation by following the ontogeny of the ultimate gene products, the polypeptide chains which make up the structural proteins of the lens, the crystallins. Such crystallin analysis requires the use of techniques in which non-covalent bonds are abolished in order that the subunits can be separated from each other. The development of a method of poly-acrylamide gel electrofocusing in 6M-urea is described. Resolution was improved by admixture of ampholytes from narrow pH ranges rather than from a single, wide pH range. Direct comparison of this technique revealed superior resolving power and preproducibility to that of polyacrylamide gel electrophoresis of crystallin samples. It was concluded that for crystallins in particular, similar electrophoretic mobilities do not necessarily indicate antigenic or structural similarities. Immunological techniques were used to identify crystallin fractions of various degrees of purity, obtained by gel filtration and electrophoretic methods, and it proved possible to identify directly the major subunits of chick alpha and delta crystallin, isolated by virtue of their isoelectric point.

In order to complete the classification of subunits, particularly those of the beta-crystallin class, a method was developed for prolonged electrophoresis in agarose. Fractions from the electropherogram, identified immunologically, were submitted to subunit analysis by isoelectric focusing. Data from this analysis, together with that obtained by electrofocusing of crystallins in non-dissociating conditions, allowed the classification of all major subunits revealed by the gel electrofocusing technique.

The range in molecular weights of crystallin subunits was also

investigated by sodium-dodecyl-sulphate (SDS) polyacrylamide electrophoresis. The results were in good agreement with other recent analyses using similar techniques and suggest that previous values in the literature are underestimates.

With the direct identification of each major polypeptide now possible, the ontogeny of polypeptides in the embryonic chick lens was reinvestigated using the isoelectric technique. The subunit analysis was in good agreement with other authors' immunological data on chick lens ontogeny. The bulk of crystallin components could be found in the 4-day embryo. Direct evidence was found for a changing composition of the delta-crystallin in development. A double-labelling procedure, employing ^{14}C and ^{3}H amino-acids was used to indicate embryonic polypeptides, whose synthesis was relatively unaffected by the antibiotic actinomycin D. Conventionally, persistent protein synthesis in the presence of this inhibitor of RNA synthesis is taken to indicate mRNA stability. Ratios of polypeptide synthesis in actinomycin-treated and control stages were calculated for a series of early developmental ages. The data revealed that direct conversion of such ratios into mRNA half-lives was not meaningful because of complexity in labelling intracellular amino acid pools. Qualitative differences in the synthetic patterns of subunits could, however, be distinguished.

The proteins associated with chick lens mRNA were also investigated since recent work has indicated that such proteins may be involved in the regulation of mRNA stability. Isolation of these components, employing zonal centrifugation, revealed polypeptides whose size range agreed closely with similar proteins from other experimental tissues. Messenger ribonucleoproteins (mRNPs) were also isolated by affinity chromatography on oligo (dT)-cellulose. The effectiveness of this

technique was proven with the well characterised mouse reticulocyte system. Molecular weight estimates of reticulocyte polypeptides isolated in this manner were in excellent agreement with published values for similar components isolated by centrifugation techniques. SDS-electrophoresis indicated that both chick lens mRNA and rabbit reticulocyte mRNA were complexed with a similar number of components within the same size range. However, gel electrofocusing revealed that the isoelectric points of these sets of proteins were entirely different. Both the complexity of these proteins and their variation within species may be greater than hitherto thought.

The findings from the subunit analyses are compared with recent evidence on bovine lens development, in the Discussion section. Estimates of the number of products representing separate loci are compared with other authors findings using less highly resolving techniques. The role of mRNA-associated proteins is reviewed in the light of this investigation's findings and other recent evidence, implicating them in early initiation events in eukaryote protein synthesis and mRNA stability. Possible means whereby differential mRNA stability can be produced are also discussed. Experiments are suggested, using data and methods described and developed in this investigation, to test current hypotheses on the nature of mRNA-protein binding, and the relationship of poly(A) shortening to mRNA stability.

PREFACE

The work described in this thesis was carried out in the Epigenetics section of the Institute of Animal Genetics, University of Edinburgh. The results within represent my own original research and have never been submitted previously, in any form, for any intermediate or higher degree.

Alan Berry .

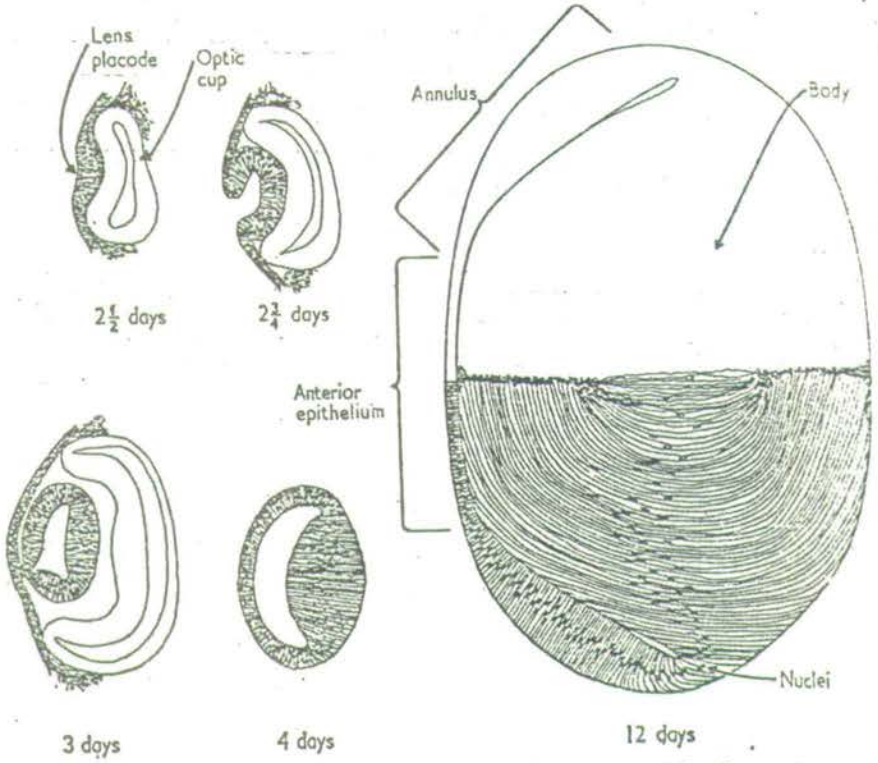

FIG. 1. Morphology of the chick lens at various stages of development. Note that the epithelium consists of two regions, the anterior epithelium and the annulus.

Chapter 1

INTRODUCTION

Cell differentiation, or cytodifferentiation, is the process which leads to the distinction between cell types in multicellular organisms. This process is generally accepted, sometimes unthinkingly, to arise as a consequence of the activity of different sets of genes in the different cell types. Many developmental biologists seek to redress our great ignorance of the mechanisms of differential gene activity but any comprehensive theory of differentiation must also consider the way in which the gene products, the proteins, influence cellular morphology and behaviour. Since differences in composition and metabolism of cell types generally involve differential enzyme activity and synthesis of specific structural proteins, it follows that the study of the regulation of protein synthesis is of key importance in understanding cellular differentiation. In this thesis an attempt has been made to analyse the changes taking place in the polypeptide composition of the chick lens during its developmental course.

The particular value of the lens as a model system for studying the biochemistry of cellular differentiation may perhaps be best realised through an understanding of its early stages of development. The optical lens is derived from the head ectoderm in response to an inducing stimulus from the underlying optic cup. The lens placode so formed bulges inwards to form a sac which separates completely from the head epidermis to form a hollow ball of cells (see Reeder and Bell, 1967; for a diagrammatic representation of the morphology of the chick lens at various stages of development). The layer of epithelial cells which make up this hollow ball are at first similar to each other. Then the posterior epithelial cells elongate rapidly until they have filled up the lumen of the ball. These elongated cells, termed the primary lens fibres, quickly stop growing.

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The bulk of the lens subsequently arises from fibres that differentiate from the anterior monolayer of epithelial cells (Davson, 1949). These cuboidal epithelial or stem cells begin to divide and their offspring are displaced posteriorly to a non-dividing, transitional zone in the epithelium known, in some species, as the annulus or annular pad. At the equatorial region of the lens these epithelial cells begin to elongate and differentiate to form fibres, the secondary lens fibres, wrapping in concentric layers around the primary fibre cells.

The newly formed fibres laid down at the equatorial region of the lens (van Heyningen, 1969). Since these fibre cells are produced continuously throughout life the fibre cells formed during the earliest embryonic growth occupy the centre of the adult lens, whilst the cortical region represent those parts laid down most recently. The entire tissue is enclosed by a non-cellular capsule, composed of collagen,-like proteins, and mucopolysaccharides secreted by the epithelial cells (reviewed in Coulombre, 1965). Since the lens is composed of a single cell type in various stages of differentiation and as no cells are lost, at any stage it embodies its own whole developmental history (Clayton, 1970). The lens is one of the few vertebrate tissues derived from a pure line of cells (Papaconstantinou, 1967) which is a major advantage for studies on cellular differentiation, since admixture with cells from other tissures is avoided.

Thus it should be clear that by comparing different regions of the lens a spectrum of biochemical changes can be studied that reflects the process of cellular differentiation. Hence one can compare the actively dividing cells of the anterior epithelium, the non-dividing cells in the transitional region of the equatorial zone or the elongated non-dividing fibre cells of the lens body.

On the basis of its developmental history alone, the lens appears as a highly suitable system for studying both the biochemistry of cellular differentiation and the process of ageing. However as pointed out by Clayton (1970) the vertebrate lens has a number of other features which increase its suitability for studies of differentiation. The lens is an a vascular tissue, with neither blood vessels or stroma. With such an isolated tissue animals are not able to develop tolerance to their own lenses. Consequently highly potent antisera to the major lens components can be developed, capable of detecting even the most conserved of antigens, whilst as a corollary, many of the most powerful immunological methods can be used to analyse lens composition and development.

The lens is also an exceptional organ in that some 30-40% of its wet weight consists of protein. Whilst possessing a range of enzymes to be expected in any metabolising tissue, some 80-90% of this protein consists of non-enzymatic structural protein, the crystallins. This high protein content is of obvious advantage to biophysicists and biochemists wishing to elucidate the structure and properties of complex macromolecules and the subsequent effects of age. Such a specialised tissue, synthesising only a small number of different proteins is also clearly a rich source for specific and pure messenger RNAs (mRNAs) that can be compared at different periods of development.

The structure of the lens is remarkably similar in all species and this uniformity extends, ~~largely~~ both to the general processes of development and the biochemistry of the fibre cell. These advantages conferred by the development and composition of the lens are supplemented by the vast amount of information revealed by morphological and embryological studies, (see for example, Lopashov and Stroeva, 1961; Coulombre, 1965).

Together with the benefit of a well documented developmental history, lens tissues can show a number of remarkable properties of interest to the

developmental biologist. Cells from the lens epithelium of the chick embryo, when cultured in medium containing serum, appear to undergo changes characteristic of epithelial cells differentiating into lens fibres *in vivo* (Philpott and Coulombre, 1965; Piatigorsky et al. 1972; Craig and Piatigorsky, 1973). Parallel to these achievements of *in vitro* analysis has been the successful isolation of calf lens crystallin mRNA (Mathews et al. 1972; Berns et al. 1972) and chick lens crystallin mRNA (Williamson et al. 1972; Zelenka and Piatigorsky, 1974), reported during the course of the work described here. Alliance of the techniques employed in these investigations makes it possible, in principle, to analyse mRNA metalbolism during *in vivo* and *in vitro* differentiation of lens tissue.

The versatility of the lens as a test system is further underlined by the fact that lenses or lentoids can be formed from a variety of tissues under experimental conditions (reviewed by Clayton, 1970) for example in the tadpoles of the toad *Xenopus laevis*, following lentectomy either iris, retina or corneal cells can give rise to lentoids after dedifferentiation. Recently the first substantial evidence demonstrating a switch of vertebrate cell types in clonal cell cultures, has been presented in this field of research (Eguchi and Okada, 1973). As predicted from studies of amphibian lens regeneration *in situ*, proliferating cells from the pigmented retina of chick embryos were shown to be capable of forming lens-like structures in culture. Clearly these types of studies should allow eventual analysis of the activation of dormant genes and make possible the cataloguing of developmental effects brought about by the changes in gene expression.

These are then the advantages offered by the lens as a model system for cytodifferentiation studies. What, however, are the particular features that mark the differentiation of the fibre cell from the epithelial cell?

Morphological and Ultrastructural Changes during the Formation of Lens Fibres.

The morphological changes occuring during the differentiation of lens fibres from epithelial cells have recently been illustrated by a comparison of cells in different regions of the intact lens of the 6-day chick embryo (Piatigorsky et al. 1972). These results have been summarised in Table 1 to outline the basic changes in morphology and ultrastructure that occur during the formation of lens fibres. Though derived from studies on the chick embryo the main features revealed are typical for many other species, including mammals.

The most striking features are the considerable elongation of the cells and the gradual loss of many of the cell organelles. Thus the density of mitochondria and rough endoplasmic reticulum decrease during the formation of lens fibres. Endoplasmic reticulum may not be present at all in older fibre cells (Mandel, 1969). Mitochondria too appear to be absent in fully differentiated fibres and the energy production of the lens body is derived from anaerobic glycolysis leading to the production of lacate (van Heyningen , 1969). As the fibre cell matures in amphibians and is displaced towards the centre of the lens the polysomal population falls off markedly (Eguchi, 1964; Karasaki, 1964). These reports confirmed earlier electron microscopic studies of mammalian lens noting an increasing sparsity of ribosomes in moving from the peripheral regions towards the centre of the lens (Wanko and Gavin, 1959).

Another major morphological feature of fibre differentiation is the inactivation and eventual loss of the nucleus (Hanna, 1965; Modak and Perdue, 1970).

The DNA of the nucleus is completely broken down eventually, Wannemacher and Spector (1968) could detect no indication of DNA in the core (the inner 25%) of lenses from young calves.

cell type	Anterior Epithelium	Equatorial Epithelium	Fibre Cell
cell shape	cuboidal	columnar,unoriented	elongate, parallel cylinders
cell thickness	14 u	55 u	(approx) 100 u
cytoplasmic packing	dense	less dense	decreased overall
granular endoplasm	plentiful	reduced in amount	little, perinuclear
mitochondria	numerous	reduced in amount	very few
ribosomes	free	some polysomes	some polysomes
mitoses	some	some	none reported
nuclei	large, folded	oval	ellipsoid
nuceoli	prominent	-	-
microlubules	few, randomly scattered	longuitudinally aligned, usually near cell surface	invariably aligned near cell surface.
amorphous granular material (crystallins)	-	-	present

Table 1. Morphological and ultrastructural changes occuring during the differentiation of lens fibures from epithelian cells, illustrated by comparison of cells in different regions of the intact lens of the 6 day chick embrye (Data from Piatigorsky et al.1972). (See also Papaconstantinou, 1967).

As indicated in Table 1, there is a decrease in mitotic rate with fibre differentiation. In general the rate of cell division progressively decreases during development and ageing. Bovine fibre cells of all ages cease to divide, whereas the embryonic epithelial cells are actively dividing and adult epithelial cells will remain in the G1 phase of the cell cycle indefinitely (Papaconstantinou, 1967). A similar situation exists in chick embryos. The rate of DNA ~~incorporation~~ synthesis and cell division drops first in the primary fibre region (Modak et al. 1968). Although initially mitosis can be observed over the entire anterior epithelium (Modak et al. 1968) it eventually is restricted to only the equatorial zone (Hanna, 1965). Lens epithelium can be triggered to re-enter mitosis by physical or chemical injury (Srinivasan and Harding, 1965).

As differentiation proceeds there is also a gradual alignment of longitudinally oriented microtubules along the inside of the cell membrane. It has been suggested that these microtubules may be required for cellular elongation but are not necessary for maintaining the elongated state since developing cells retain their shape when microtubules are disrupted with Colcemid (Pearce and Zwaan, 1970). This drug does prevent the formation of primary fibres in the lens placode (Pearce and Zwaan 1970). Similarly colchicine and vinablastine, which also dissociate microtubules, prevent the elongation of cultured epithelium cells (Piatigorsky et al. 1970) but again the authors found that where cells have begun to elongate in culture, they do not lose their shape upon treatment with colchicine. Clearly there are other intrinsic factors involved in cellular elongation other than microtubules but the initial orientation of microtubules along the inside of the cell membrane may be <u>essential</u> for the formation of lens fibres, (Piatigorsky et al. 1972).

The gradual increase in amorphous granular material (see Table 1) was also noted by Wanko and Gavin (1959) and is possibly due to the accumulation of crystallins.

To summarise, during fibre differentiation a decrease in organelle density, polysome loss and an alignment of longitudinally oriented microtubules along the inside of the cell membrane, all take place. At the same time the cells show a gradual loss of ability to divide that is followed eventually by their enucleation.

Macromolecular events occuring during fibre differentiation

Some of these events are obvious corollaries to processes described in the previous section. Thus the DNA content of young embryonic fibre cells is constant whereas in maturing fibre cells DNA disappears after the degeneration of the nucleus (Wannemacher and Spector, 1968). Concomittant with the indentified breakdown of polysomes is the loss of RNA, shown in the rabbit lens, during fibre cell maturation, due to a depletion of microsomal RNA (Dische et al. 1962). The low molecular weight RNA that persists in bovine fibre cells appears to be primarily tRNA, rather that rRNA breakdown products (Zapisek and Papaconstantinou, 1973). Thus the decrease in total RNA seen in vertebrate lens development appears to be due mainly to the eventual degradation of ribosomal RNA. Prior to this actual loss of material, changes occur in the maturation process of rRNA. Bovine lens cells that are capable of division show a significant chase of radioactive label into 28s and 18s rRNA but in non-dividing cells only 45s and 30s ribosomal precursor RNA is synthesised and no further processing of rRNA occurs (Papaconstantinou and Julku, 1968). This appears as an obvious economy to avoid recycling of rRNA components, prior to their progressive degradation.

The most obvious biochemical event, however, in lens differentiation is the synthesis of the crystallin polypeptides but there are some remarkable and unexpected changes in protein synthetic patterns in the older fibres of certain species. Although RNA synthesis in general falls, a type of structural protein, gamma-crystallin, accumulates in large amounts in amphibian lens fibres, firstly in the primary fibres and then

Table 2.

Summary of major molecular events reported to occur in the differentiation of the lens fibre cell.

Component	Remark(s)
DNA	disappears in fibres after nucleus disintegrates
Polyribosomes	general decrease in number from epithelium towards lens centre
r RNA	maturation process inhibited, eventually degrades fully
t RNA	remarkably persistent in fibres, distribution similar in epithelium
Protein	a) initial, conspicuous synthesis of crystallins. b) general decrease (no synthesis at all in lens core of young calves) c) in some species, late synthesis of crystallins characteristic of fibre cells.
m RNA	apparently stabilised in fibre cells (on basis of actinomycin studies)
RNA polymerase) RNAse) RNAse inhibitor)	virtual total decrease of activity in fibre cells.
Histones	Initially lysine rich fraction decreases

spreading to the secondary fibres (Takata et al. 1964). Synthesis of gamma-crystallin in the bovine lens also appears to be characteristic of fibre development (Papaconstantinou, 1965). This apparent switch in template activity to produce gamma-crystallin, when RNA synthesis is generally decreasing, suggests that gamma-crystallin could be involved in some way with the final suppression of genome transcription (Yamada, 1969) but no mechanism for this type of inactivation has ever been proposed. At least in these species, gamma-crystallin synthesis seems fully indictive of terminal cell differentiation. McDevitt (personal communication) has never observed a mitotic figure in lens cells from Rana pipiens containing gamma-crystallin.

Changes in crystallin composition occur with increasing age of the animal. Thus differences between the epithelium, the cortex and the nucleus of the lens have been reported by many authors (reviewed in Clayton, 1970, 1974). The evidence that the subunit composition of each class of crystallins in the chick lens may change with age is reviewed in detail in later sections. However in general the epithelium of older animals does not synthesise crystallins of the identical range as epithelium from younger animals. For example Rabaey (1962) found one structural component (delta-crystallin) was not present in the outer layers of the adult chick lens but predominant in early embryonic life.

Whilst the youngest cells laid down may show differences in template activity from older fibres, concomittant with the polysome breakdown and ribosomal RNA degradation in the oldest nuclear fibres, there is a total shutdown in protein synthesis. Spector and Kinoshita (1965) could only detect a very low level of incorporation of amino-acid into the inner 50% of young calf lens. When this group re-examined this finding they could detect essentially no activity in the protein of the core region, representing the inner 25% of the lens (Wannemacher and Spector, 1968). This decrease in protein synthesis is in marked contrast to young cells

where such activity is persistent. Thus in the 10-day embryo of the chick autoradiographic evidence has been presented showing incorporation in the core of the lens after 1-hour exposure to C^{14}-leucine, whilst even after actinomycin D treatment the C^{14}-leucine grains are evenly distributed, throughout the core (Reeder and Bell, 1965).

The last mentioned results, together with a number of other early reports, indicated that the synthesis of proteins on lens polyribosomes was inhibited by actinomycin D to a much smaller extent than those of many other tissues (for example see Scott and Bell, 1964; Spector and Travis, 1966). This antibiotic inhibits RNA synthesis by binding to DNA and impeding the action of DNA-dependent RNA polymerase, with usually a subsequent inhibitory effect on protein synthesis (Goldberg et al. 1963; Reich and Goldberg, 1964). The tacit assumption made in many early investigations was that the decrease in protein synthesis was due to the mRNA, present before actinomycin D treatment, gradually decaying without being replaced. Thus it has been presumed that where there is protein biosynthesis in the presence of this inhibitor of RNA synthesis then a stable mRNA must exist. Based upon this premise a number of additional early reports strengthened the concept that a further characteristic of the terminally differentiated fibre cell is the possession of stable mRNA (Spector and Kinoshita, 1965; Reeder and Bell, 1967; Stewart and Papaconstantinou, 1967 a,b). Thus the lens system appeared, on the basis of these results with actinomycin D, to be an ideal experimental means to investigate factors controlling the stability of mRNA and certain approaches to this problem have been made herein (Chapters 10-14). However it may be more valuable to interpret these and other results on this subject, after fuller examination of the concept of differential mRNA stability, particularly since some recent results, published during the course of this work, question the premises

and rationale of the earliest experiments (review section II of this Introduction).

Other molecular aspects of differentiation of lens fibres have been presented (Virmaux et al. 1969) but the experimental results have not been published to date. These authors claim that RNA polymerase activity decreases and disappears from the fibres, in parallel with similar decreases in RNA activity and RNA inhibitor. These authors also claim that the first step in fibre differentiation is a decrease in amount of the lysine-rich histone fraction. Since other authors have reported that lens RNase levels are too low for detection (Zigman and Lerman, 1965; Swanson et al., 1967) it would be of major value if the results claimed in Virmaux et al. (1969) were substantiated by published data. Maione et al. (1968) could find no measurable RNase activity in normal human lenses but RNase activity was particularly high in the cortex of cataractous lenses, together with a marked decrease in heavy polyribosome aggregates. Lower levels of RNase activity were recorded in posterior and nuclear cataracts. Bloemendal (1969) has also claimed that in contrast to lens epithelium, RNase is extremely low in the fibres.

A summary of the major molecular events reported to occur in the differentiation of the lens fibre cell is shown in Table 2.

Additional biochemical events occuring during fibre differentiation

The gradual loss of mitochondria as epithelial cells differentiate into fibre cells, coupled with a transition from replicative to non-replicative state is bound to be reflected by considerable metabolic differences between the younger (outer) and older (inner) layers of the lens. In general the epithelial cell is able to utilise mitochondrial respiration, whereas the fibres become more dependent on active anaerobic glycolysis converting the bulk of their glucose to lactate. The change over from a highly oxidative to anaerobic metabolism together with the changes in mitotic state occuring in fibre differentiation produce several phenomena

of interest to the developmental biologist as well as to the classical biochemist. However rather than review the piecemeal situation that existed when this investigation began, it may exemplify understanding to relate also some of the more recent evidence in order to present a more coherent pucture.

Metabolic changes, introduced by necessity with differentiation, can of course be compensated for, if enzymes of high specific activity can be enhanced or sustained in the epithelium. Enzymes concerned with active uptake of materials may perform general functions for the whole of the lens (Shapiro, 1968b: van Heyningen, 1969). The wider spectrum of enzymes in the epithelium may also be able to release particular metabolic blocks that plague less well endowed cells, by utilising additional biochemical pathways. Evidence has recently been presented that whilst the regional activities of ketohexinase decreases in the order, epithelium, cortex, nucleus the specific activity of ketohexinase in the epithelium actually increases with age (Ohrloff et al. 1974). The authors point out that other enzymes involved in this sorbitol pathway do not show the expected decrease in activity with age that is characteristic of the majority of enzymes involved in carbohydrate metabolism.

Another feature demonstrated during fibre differentiation is change of iso-enzyme patterns, presumably determined by constraints imposed by a decreased energy metabolism or loss of replicative ability. The overall picture is one of relatively complex types of control, and variation in the epithelium coupled with a simpler system in the older lens fibres, that often culminates in no detectable activity at all in the innermost nucleus. Mammalian lenses, for example, contain two distinct iso-enzymes of hexokinase, types I and II, present in a complex mixture of soluble and insoluble forms (Chylack, 1974). In the nucleus virtually all of the iso-enzymes are soluble and are type II. Whilst there is no hexokinase activity in the capsule, some 35-80% (depending on species) of the total

hexokinase in the epithelium itself is found in the particulate fraction. Although the binding site has not been established, it is striking that some 30% of this insoluble hexokinase is in a latent or inactive state. Virtually all of this bound enzyme is the type I iso-enzyme. The type II iso-enzyme is extremely thermolabile, in the absence of glucose, irrevesibly in enzyme preparations and reversibly in the intact lens (Chylack, 1973). The correlation between low glucose levels and the lability of the type II iso-enzyme suggests that the differences in epithelial and fibre iso-enzymes patterns are dictated by metabolic factors, although this point will remain conjectural until the kinetics of reaction of the two enzymes types have been fully compared. The existence of the latent form of hexokinase in the epithelium is puzzling. (Conceivably it could be brought into play when metabolic activity and ATP requirements are high). More importantly the author points out that the presence of soluble and latent hexokinase in the epithelium imply a greater sensitivity to environmental changes, together with a greater flexibility of response to these changes.

Mitotic state rather than metabolic considerations may influence the changes in the types of lactate dehydrogenase (LDH) iso-enzymes during differentiation of the fibre cell (Stewart and Papaconstantinou, 1966). The most cathodal form of the tetramer iso-enzyme (LDH-5) predominates in epithelial cells but LDH-1 (the most anodal form) predominates in the fibre. A similar displacement towards LDH-1 occurs with increasing age in other tissues but the persistance of LDH-1 remains puzzling since it is usually found in tissues of high oxidative capacity. Since a predominance of LDH-5 can be regained by placing adult epithelial cells in culture, whilst the synthesis of LDH-1 subunits is favoured in the stationary phase of a logarithmic growth cycle, the authors suggest that the regulation of LDH subunit synthesis may be associated with the mitotic state of the cell.

The synthesis of major amounts of subsequently metabolically stable

components is another solution to the problems posed by the decreasing catabolism of the lens with time. Lest the impression has been created so far that differentiation of the lens fibres from the epithelial cell is one of wholesale degeneration of cellular components, it may be worth recalling that one of the striking features of this process is the massive increase in cell length. This requires a tremendous increase in the plasma membrane that can only be provided by a very active biosynthetic apparatus. The plasma membrane appears to be highly stable since it shows a lifelong maintainance in the fibre cells (Broekhuyse et al. 1974). A number of studies have been made of the metabolism of the phospholipid sphingomyelin, a membrane component, in view of the large increase in the relative concentration of sphingomyelin during differentiation from epithelial cells (Broekhuyse, 1969). A recent study has examined the subcellular distribution of sphingomyelinase and many other enzymes in the epithelium, equator and cortex of the calf lens (Roelfzema et al. 1974). After tracing marker enzyme activities characteristic of mitochondria, microsomal membranes, plasma membranes, lysosomes and cytosol, the authors made two general points about lens metabolism. Firstly the determined enzyme activities were very low in all cellular regions (some enzyme activities were a hundred times lower than those in rat liver). Secondly, during the course of differentiation, the absolute activities of the enzymes, in units per gram tissue, decreases in the order epithelium to equator to cortex, probably because the number of subcellular organelles is reduced per tissue. In common with other enzymes held to be characteristic of lysosomes, sphingomyelinase showed a cellular distribution between the mitochondrial marker enzymes on one hand and the microsomal and plasma membranes on the other. The authors thus propose a lysosomal origin, although lysosomes have not, as yet, been identified unequivocally in the lens. Swanson et al. (1967) presented some histochemical evidence for lysosome-like particles. A protease, often characteristic of lysosomes, has been detected in human senile cataractous lens, capable of hydrolysing bovine lens proteins (Swanson and Nichols, 1971).

Sphingomyelins themselves are extremely similar in the calf lens cortex and nucleus but there is a marked increase in long chain fatty acids during differentiation from the epithelial cell to the cortex fibre (Broekhuyse et al. 1974). Cow lens sphingomyelin is also similar to calf lens sphingomyelin suggesting that the lipid remains metabolically stable during the ageing of the lens. The data of this group indicate that synthesis of sphingomyelin occurs only in the equator of the lens and that sphingomyelin in the nucleus and cortex remains metabolically stable. The levels of sphingomyelinase are very low in the older parts of the lens (Roelfzema et al. 1974), the inertness of the phospholipids presumably contributing to the physical and metabolic stabilisation of the persistent plasma membrane.

To summarise at this point it appears that the fibre cells with an increasing dependence on anaerobic glycolysis, paralleling the gradual disappearance of mitochondria from the differentiated fibre, can exhibit a number of biochemical sophistications. Firstly it can simply rely on enhanced or sustained enzyme activity in the epithelial cells, perhaps involved in minor metabolic pathways, to prevent the accumulation of substrates that might otherwise occur in the fibres because of their decreased range and lower activities of enzymes (e.g. ketohexinase activity). Secondly iso-enzyme patterns can be selected that, presumably, enhance activity in the particular conditions of the fibre cell. The choice of iso-enzyme used may depend upon relative efficiencies in anaerobic conditions or be related to the non-dividing state of the cell. This choice may involve the depression of the synthesis of particular iso-enzyme subunits. The synthesis of subsequently metabolically and physiologically stable components in massive amounts by equatorial cells, obviously cuts down the amount of biosynthsis that has to be carried out in the fibre cell (e.g. sphingomyelin synthesis). Finally although, in general, the order

of absolute activities for virtually all enzymes studied decrease in the order epithelium to cortex to nucleus, exceptions to this rule do occur. Thus the activity of inositol - I - phosphatase is three times higher in the nucleus than either the epithelium or cortex of calf lenses, in contrast to the distribution of free inositol which is 60% higher in the epithelium and capsule that the rest of the lens (Kabasawa et al. 1974). Unfortunately the significance of the high enzyme activity in the nucleus is not known.

One more major but separate feature of the differentiation of fibre cells must be mentioned. The production of lens capsul proteins seems to stop immediately after the formation of fibre cells. The convincing biochemical and genetical evidence that the epithelial cells synthesise the capsule of the lens, the major components of which are collagen and carbohydrate has been reviewed by Clayton (1970). The intensity of ~~carbohydrate~~ protein synthesis appears to be greatest at the equatorial zone (Young and Fulhorst, 1966) and it is tempting to speculate, by analogy with sphingomyelin synthesis, that glycoproteins involved in membrane formation are produced at this time, prior to the massive increase in cell surface that occurs in fibre differentiation. It is still rather a vexed question as to whether all biosynthetic events can occur in the fibre, or whether some components are produced only when the cells are still in a replicative state. The data on sphingomylin synthesis suggests that some dependence of epithelial synthetic activity may be necessary but it seems likely that the cessation of synthesis of many components will be gradual, as the biosynthetic capacity of the fibres decreases with consistent organelle loss. What is striking is that in addition to synthesis of the whole gamut of enzymes required for any rapidly growing cell, the epithelium simultaneously produces two sets of structural protein; crystallin and capsular protein. The factors influencing the depression of synthesis of capsular constituents are unknown, but Zwaan (1969) has pointed out that

this may only occur when the fibre cell loses contact with the surface of the lens. Capsular material such as collagen is still synthsised by lens epithelial cells in culture (Green et al. 1966).

It is also known that epithelial synthetic activity can also be affected by products of the previously formed fibres. Thus mitotic inhibitors have been extracted from the cortex and nucleus of rabbit lenses (Froomberg and Voaden, 1966; Voaden, 1968). Synthesis of capsular components may depend on replicative ability: alternatively the build up of other types of inhibitors in the inner layers of the lens may influence the capacity of outer layers to produce these components.

EXPERIMENTAL APPROACH

Bloemendal et al. (1962) first demonstrated that a main fraction of the bovine lens, alpha-crystallin, was composed of subunits. When alpha-crystallin was dissolved in 7 M urea a sharp decrease in sedimentation constant occured, with an estimated drop in molecular weight from 800,000 to about 25,000. The authors also demonstrated that the urea-treated protein can be separated into a number of fractions on starch or polyacrylamide gels containing high concentrations of urea. Bjork (1964) confirmed these observations and presented evidence that the subunits differed in electrophoretic properties and amino-acid composition. Later results including treatment with other reagents also capable of breaking down non-specific, non-covalent bonds between polypeptides, reagents such as guanidinium hydrochloride and sodium dodecyl sulphate (SDS) confirmed the subunit hypothesis (Spector and Katz, 1965; Bloemendal et al. 1965).

Subsequently the isolation and characterisation of these subunits have been pursued in numerous laboratories employing various techniques and there is general agreement that there are only four different polypeptides in bovine alpha-crystallin (e.g. Bjork, 1968; Bloemendal and Schoenmakers, 1968; Schoenmakers and Bloemendal, 1968; Palmer and Papaconstantinou, 1968; Stauffer et al. 1973).

Zwaan (1963) found chick alpha-crystallin separated into 5-6 bands when subjected to urea-electrophoresis. Clayton and Truman (1967) showed that all of the soluble proteins of the chick lens could be dissociated by urea and guanidinium hydrochloride. These authors also presented immunological evidence to indicate that all of the major classes of crystallin in the chick lens were probably composed of hetro-polymers, that is built up of a number of different subunits. Evidence is presented below, in detail, that the chief classes of chick crystallin are each composed of subunits but the significance of such a subunit structure in studies on the differentiation of the lens should be stressed at this point, since it forms a theoretical basis of the investigations reported here.

If the differentiation of the lens fibre is to be considered as a system involving the activity of a number ofdifferent genes, then it is the syntheses of the individual subunits that must be followed. The ennumeration and comparison of subunits is obviously vital to any estimation of the number of related genes active in the tissue. Consequently studies of the rate of protein synthesis at different stages of development, any analysis of evolutionary changes in the crystallins or the relationships between species must be made in terms of the subunits of the major classes of protein. This investigation has tried to meet this prequisite for a high resolution method for the analysis of crystallin subunits by the development of a gel electrofocusing technique employing dissociative conditions (Chapter 3). With this technique an attempt has been made to analyse the changes in subunit composition of the lens during its developmental course (Chapter 14). The particular value of this kind of approach has been previously stressed by Clayton (1970) and Truman et al (1972b).

Clearly any estimate of the number of related genes active in the development will depend upon accurate identification of the number of

separate peptide chains synthesised. One must therefore be aware that such an estimation of numbers may be complicated by post-synthetic modifications of the peptides (Clayton, 1970). Thus processes such as deamination, acetylation, cross-linkage, proteolysis or oxidation may arise during the extraction procedure or occur naturally within the lens. Artifactual changes may also be introduced by the isolation technique itself, such as carbamylation or precipitation or proteins. However with such forwarning, many possible sources of artefacts can be eliminated.

The work performed here was carried out on chick lens and the properties and nomenclature of the major crystallin classes are discussed below, in a review of work generally published prior to the beginning of this investigation. However where later work has considerably clarified particular aspects of crystallin properties, this has been included to aid understanding.

THE CRYSTALLINS OF THE CHICK LENS

The soluble proteins of the mammalian lens have long been generally classified into three groups, the alpha, beta and gamma-crystallins, determined by their electrophoretic mobility at an alkaline pH. Thus in free electrophoresis alpha-crystallins migrate furthest towards the anode, while the gamma-crystallins migrate furthest towards the cathode. The proteins of the chick lens are also generally considered to be assignable to three major groups of crystallins but unfortunately no uniformity in nomenclature of the chick crystallins has been fully agreed upon, to date. The nomeenclature used here is that of Zwaan and Ikeda (1968) who used the Greek letters alpha, beta and delta to designate these three groups. This nomenclature has the advantage of emphasising the immunological cross-reaction of the chick alpha and beta-crystallins classes with mammalian alpha and beta-crystallins (Zwaan and Ikeda, 1968) whilst stressing also the separate nature of the delta-crystallin class which is found in

birds and some reptiles but is absent in mammals and amphibians.

CHICK ALPHA-CRYSTALLIN

The alpha-crystallin of chick lens can be readily characterised as the fraction of highest electrophoretic mobility (in alkaline conditions) and highest molecular weight. It appears to be al least partially homologous with mammalian lens alpha-crystallin (Maisel, 1964; Swanborn, 1966; Waley 1969). Its molecular weight is however, markedly lower than that of bovine alpha-crystallin. Although the size distribution of bovine alpha-crystallin is complex (Spector et al. 1971) the authors found by a combination of gel filtration and equilibrium centifugation that the major population has a molecular weight ranging from approximately 700,000 to 1,000,000. This is in good agreement with earlier work by Hoenders(1965) who obtained a value of 840,000 using analytical ultracentrifugation and Zwaan (1968) who estimated its size as 900,000 by employing a method dependent on electrophoresis in polycrylamide gel. In contrast Hoenders (1965) found that the alpha-crystallin of the chick has a molecular weight of 470,000, while Zwaan (1968) separated three different species of this class with molecular weights estimated at 400,000, 350,000, and 60,000. Using a gel filtration method Truman (1968) also discovered evidence of polydispersity of molecular weight in the alpha-crystallin group and resolved three major components by polyacrylamide electrophoresis. Immuno-electrophoresis also revealed three concentric precipitin arcs (Truman, Clayton and Campbell, 1967). Osserman tests indicated three separate precipitin lines merging with the alpha-crystallin arc in embryos of $4\frac{1}{2}$-5 days (Truman et al. 1972 a). Thus there appears to be a general correspondence of results indicating that alpha-crystallin as separated from chick lens consists of at least three different molecular species. These differences observed in the molecular weight of the species may be due to factors other than primary structural changes since bovine alpha-crystallin, which increases in molecular weight with age, does not appear

to alter with time in amino-acid composition (Stauffer et al. 1973; Li, 1974).

As calculated by quantitative immuno-electrophoresis the relative proportion of alpha-crystallin in the chick lens remains reasonably constant from embryos of about 16 days to adulthood (Truman et al. 1972 a). However these authors believe that proportions of the antigen determinants of alpha-crystallin may change during development, since the relative positions of the precipitin lines which react with adult alpha-crystallin vary at different stages. The precipitin lines formed by the embryonic lens gradually become closer in ages above $4\frac{1}{2}$ days old. In addition quantitative immuno-electrophoresis revealed that the precipitin peak formed by alpha-crystallin is more complex from this age onwards. Clayton (1969) obtained direct evidence that the subunit structure of chick alpha-crystallin changes in composition during development. Polyacrylamide electrophoresis in dissociating conditions revealed two major bands and two minor bands in re-run of alpha-crystallin regions isolated from 13 day old embryos. 18 day embryos showed an additional faint band. However in the young adult an additional major band of higher mobility was observed together with four to seven minor bands. Rana and Maisel (1969) also found that the composition of chick alpha-crystallin changed during development, the adult lens showing three major subunits, the embryonic lens only two. The composition of the alpha-crystallin in the adult epithelium resembled that found in embryos and the authors associated the appearance of this third subunit with the differentiation of the fibre in the older mammal.

Truman et al. (1972 a) could find no evidence for the appearance of any different antigenic determinants after the first appearance of alpha-crystallin antigens in (about) 4 day old embryos, implying that any newly appearing subunits are very similar antigenically to the

earliest synthesised subunits. One possibility is that subunits appearing late in development may not be products of direct genetic translation but arise by slow post-translational conversion processes of other subunits, a phenomena known to occur in bovine alpha-crystallin. Thus although changes in the subunit composition of bovine alpha-crystallin occur with age (Schoenmakers and Bloemandal, 1968; Palmer and Papaconstantinou 1968, 1969), one subunit (alpha A_1) appears to arise as the result of a slow biochemical conversion from another (alpha A_2) - (Palmer and Papaconstantinou, 1969). No significant uptake of radio-active leucine in subunit alpha A_1 could be detected in a 7 hour incorporation period although this polypeptide has approximately the same number of leucine residues as do the other alpha-crystallin subunits (Delcour and Papaconstantinou, 1972). Examination of extracts of successive concentric layers from the cortex to the nucleus by iso-electric focusing in polyacrylamide gels containing urea, revealed four main bands alpha A_1, alpha A_2, alpha B_1 and alpha B_2 but also several additional polypeptides in minor amounts (van Kleef and Hoenders, 1973). Nine polypeptides chains in bovine alpha-crystallin and their particular distributions could be identified by this technique (van Kleef, Nijzink, and Hoenders, 1974). Hoenders' group suggest that these age related changes occur partly through deamidation and partly through a fragmentation of polypeptides into well defined smaller ones. Deamidation of polypeptide chains is the commonest source of micro-heterogeneity in protein populations (Williamson (A.H.) et al. 1973).

By analogy with these studies on bovine alpha-crystallins, changes in the subunit composition of chick alpha-crystallin with time may reflect similar process. Thus changes in immuno-electrophoretic patterns, electrophoretic mobilities and sedimentation values may reflect modifications of pre-existing subunits rather than <u>de novo</u> synthesis of additional subunits.

This investigation has amongst other things, tried to identify and enumerate the number of alpha-crystallin subunits in the chick lens and compare their patterns of incorporation of labelled precursors during the early stages of development. This has been approached as follows:

(1) By using various partially purified crystallin fractions, obtained by gel filtration and polyacrylamide electrophoresis, an attempt has been made to identify and ennumerate the total number of alpha-crystallins in the chick lens by means of gel electrofocusing in dissociative conditions, (Chapters 4 to 6).

(2) Isolated portions of the agar electrophoretic spectrum, including the alpha-crystallins have been compared by immunological analysis and subunit composition studies (Chapter 6).

(3) The ontogeny of these subunits, their relative rates of incorporation of amino-acids and their mRNA stability, as determined indirectly by actinomycin D studies, are reported in Chapter 14.

These approaches have also been utilised in studies on delta and beta crystallin subunits and reported in the same chapters.

CHICK DELTA CRYSTALLINS

This class of crystallins is immunologically quite distinct from the other crystallins and is found only in birds and some reptiles. It is the major component of the chick lens first demonstrated by Rabaey in 1962. Since immuno-electrophoretic studies indicated that it was the first crystallin to appear in quantity during development of the chick lens he termed it F.I.S.C. (first important soluble crystallin). Zwaan and Ikada (1965, 1968) and Zwaan (1968) adopted the name of delta-crystallin, to emphasise its distinctiveness from other crystallin classes. Whilst most references in the literature now refer to it as delta-crystallin or FISC (review Clayton, 1970, 1974) its electrophoretic mobility in alkaline conditions is similar to that of mammalian beta-crystallins,

hence it has been termed beta-crystallin (Maisel and Langman, 1961a) and beta mobility crystallin (Clayton et al. 1968).

This fraction of the chick crystallins is intermediate in molecular weight between the alpha-crystallin and beta-crystallin (Zwaan, 1968), and, as pointed out above, was called delta-crystallin in order to emphasis that it does not cross-react immunologically with any of the major classes of mammalian crystallins (Zwaan and Ikeda, 1968). However some authors have (logically) reserved the letter delta for the protein class with the highest cathodeic mobilities of all crystallins, those found in adult fish and some amphibia (Bon et al. 1964; Bon, 1969). This group of proteins is not analogous with the delta-crystallins of the birds and reptiles.

Fortunately more agreement and consistency can be found over the developmental history of delta-crystallin than in its history of nomenclature. Most recent studies agree that in early embryonic stages delta-crystallin is among the earliest proteins found in the lens and constitutes the predominent protein of the lens in all of early development. Rabaey (1962) could detect this component immunologically after 72 hours, whilst alpha-crystallin (which did not migrate in electrophoresis in the conditions that he used) could not be demonstrated until the 6 day embryo. The first appearance of lens antigens in embryonic lens tissue has also been repeatedly studied by immunofluoresence (Zwaan and Ikeda, 1965, 1968; Ikeda and Zwaan, 1967).. This technique showed the first appearance of delta-crystallin in 2 day old embryos and of alpha-crystallin in 3½ day old embryos. These results have been confirmed by the immunofluorescence studies of Brahma and van Doorenmaalen (1971). Using a radio-immunoprecipitation technique it has been possible to show that the initial synthesis of delta-crystallin occurs in 54 hours embryos, followed thereafter by a sharply increasing rate of synthesis (Katoh and Yoshida, 1973). The results of Osserman tests on extracts of embryonic lenses indicated a slight trace of delta-crystallin in 3-3½ day old embryos.

By 3½-4 days the embryonic protein appeared fully identical, immunologically, with adult delta-crystallin (Truman et al. 1972 a).

There appears general agreement among workers that delta-crystallin represents 60-80% of the soluble protein synthesised in the lens fibres during the first few weeks of development (Rabaey, 1962; Genis-Galvez et al. 1968 b; Yoshida and Katoh, 1971 b; Truman et al. 1972 a; Piatigorsky et al. 1972, Craig and Piatigorsky, 1973). It is not however absolutely certain that delta-crystallin is the very first class to be synthesised in the lens, or indeed whether at any age one crystallin class is synthesised solely. Much early work has identified the earliest lens-specific proteins as alpha-crystallin, in embryos of 44 hours and earlier (Maisel and Langman, 1961b; Clark and Fowler, 1960; Perlmann and de Vincentiis, 1960; Kraijenhoff Sloot, 1963). Truman et al. (1972) detected a beta-crystallin in the eye region by the Osserman technique at about 50 hours, before any other crystalins. The authors suggested that there may be cross-reactivity between some beta-crystallins determinants and extra-lenticular tissues removed as contaminants with the very early tissue. Given this confusion over the nature of the crystallins synthesised during the very early stages of development and since immmunofluorescence studies only visualise accumulation products at the time of sampling, it seemed worthwhile to re-examine again the first stages of ontogeny using a highly resolving technique for protein analysis of pulse labelled lens tissue (Chapter 14).

There is conflicting evidence as to whether native delta-crystallin exists as a homogeneous species. The crucial distinction that must be resolved in any study of the ontogeny of delta-crystallin is to whether it consists of identical monomers or of two or more polypeptides with different amino-acid sequences. Clearly evidence of polydispersity, evidence that delta-crystallin represents a heterogeneous population of proteins, would favour the concept that delta-crystallin is a heteropolymer,

of different subunits. Unfortunately there is considerable disagreement on even such basic physical properties as molecular weight. Published values for both the molecular weight of native delta-crystallin and on its subunit molecular weight vary considerably. Maisel and Langman (1961a) investigated adult chick lens crystallins by sedimentation velocity centrifugation and estimated a molecular weight of 200,000 for delta-crystallin. A similar value was established for native delta-crystallin from lenses of 13-17 day old chick embryos, in a recent study employing gel filtration and sedementation equilibrium centrifugation (Piatigorsky et al. 1974). Hoenders (1965) also measured the sedimentation constant of chick delta-crystallin and obtained a value for the molecular weight of 165,000, his value for both the sedimentation constant and diffusion coefficient differing from the results of Piatigorsky et al. (1974). Zwaan (1968) using a separate technique based on electrophoresis into two concentrations of polyacrylamide, indicated polydispersity in native delta-crystallin, with a molecular weight range of 55,000 to 460,000 with a predominant species of 160,000. Truman (1968) found indications of polydispersity of molecular weight in gel filtration studies on chick lens proteins. Gel filtration techniques gave an estimate of about 150,000 to 160,000 for the molecular weight of adult delta-crystallin (Truman et al. 1971). In contrast both filtration on agarose gel and sedimentation equilibrium centrifugation revealed no evidence of polydispersity (Piatigorsky et al. 1974).

As mentioned above there is also disagreement on the subunit molecular weight of delta-crystallin. Gel filtration of delta-crystallin analysed in 7 M urea indicated a subunit molecular weight of 26,500 (Truman et al. 1971). Electrophoresis in polyacrylamide gels containing sodium dodecyl sulphate (SDS) allowed Piatigorsky et al. (1972) and Craig and Piatigorsky (1973) to estimate a delta-crystallin subunit molecular weight of about 45,000. A single sized subunit of this molecular weight has been obtained also by sedimentation equilibrium in SDS (Piatigorsky et al. 1974).

Since a full understanding of the properties and composition of the native protein is dependent on accurate estimations of subunit molecular weight, the molecular weight of delta-crystallin after dissociation has been re-examined (Chapter 9). In order to facilitate comparisons with other authors' estimates, lenses of the same source and age as those used by Truman et al. (1971) were subjected to SDS-polyacrylamide electrophoresis of superior resolving power to that used by Piatigorsky et al. (1972).

It is Piatigorsky's group that has provided the most recent and most compelling evidence that delta-crystallin is a homogeneous protein (Piatigorsky et al. 1974). Other work justifying this conclusion tends to be much earlier, employing techniques of comparatively low resolving power. Cellulose acetate electrophoresis (Genis-Galvez et al. 1968 b; Yoshida and Katoh, 1971 a) and sedimentation studies (Maisel and Langman, 1961a; Rabaey 1962) resolved delta-crystallin as a single entity. In contrast where methods of increased resolution have been employed the general consensus appears to be that delta-crystallin is a heterogeneous population of proteins. Thus Rabaey (1965) demonstrated heterogeneity by using prolonged agar gel electrophoresis. This phenomena has also been demonstrated by two dimensional polyacrylamide electrophoresis (Zwaan, 1968) and agar gel immuno-electrophoresis (Zwaan and Ikeda, 1965; Zwaan, 1968). Native delta-crystallin, purified by ion-exchange chromatography was separated into seven components, showing the same serological determinants, by iso-electric focusing in polyacrylamide gels (Bours and van Doorenmaalen, 1970, 1972). Similar results using this latter technique have been reported by Craig and Piatigorsky (1973).

These differences in results are clearly confusing. However it must be borne in mind that subunits differing only in a small number of amino-acid sequences might not be resolved by techniques that separate on the basis of molecular weight alone. Studies on re-electrophoresis into polyacrylamide gels containing urea indicated that delta-crystallin

is composed of five non-identical polypeptides (Clayton, 1969). Truman et al. (1972) employing starch gel electrophoresis in 6 M urea at pH 5.6 could resolve four bands of delta-crystallin. The acidic pH of the starch gels is much less likely to introduce artefacts by carbamylation than is the alkaline urea used in polyacrylamide gels (Stark et al. 1960). Further indirect evidence implying heterogeneity of the chick delta-crystallin molecular population can be found by inter-species comparisons. Native duck delta-crystallin has been shown to be electrophoretically very disperse, resolving into five separate bands in a short polyacrylamide run (Rabaey, 1965). Whilst chick delta-crystallin was resolved into a single diffuse region by polyacrylamide electrophoresis at pH 8.9, duck delta-crystallin was resolved into five components, each of which ran accordingly to its original mobility upon re-electrophoresis (Clayton, 1969). Thus resolution of chick delta-crystallins into a single component may yet prove to be fortuitous, reflecting difficulties of resolution rather than homegeneity of the molecular species.

In an attempt to answer the vexed question of the nature of chick delta-crystallin subunits, immunologically pure delta-crystallin has been gel electrofocused in the presence of urea (Chapter 5). In addition by developing a method for extensive (19-18 hours) agar electrophoresis of total adult crystallins, an attempt has also been made to analyse the subunit composition of the crystallins, whose composite state has been identified immunologically (Chapter 6).

CHICK BETA-CRYSTALLINS

The class of chick lens crystallins of the lowest molecular weight and the widest range of electrophoretic mobilities of all crystallins in now generally refered to as beta-crystallin. This name derives from their reported antigenic similarities to bovine beta-crystallins (van Dam et al. 1963; Zwaan, 1966; Zwaan and Ikeda, 1968, quoting unpublished data). In

immuno-electrophoresis they show a long precipitin line extending from the extreme anodal to the extrme cathodal end of the immunopherogram. Against homologous antisera this line shows several spurs, with a point of inflexion close to the centre of the delta-crystallin arc. Because of the appearance of the precipitin arc they produce in immuno-electrophoresis Zwaan (1963) referred to them as "long line" crystallin. Clayton (1969) used both the terms "long line" and beta-crystallin, other authors have also used the term "Long Line Material" (Bours, 1971; Bours and van Doorenmaalen, 1972). Some earlier references in the literature also referred to them as gamma-crystallins because of their low molecular weight and the low electrophoretic mobility of some of their members (Maisel and Langman, 1961a; Zwaan et al. 1963).

The tortuous changes in name of this crystallin class, throughout the literature, are matched only by the exceptional complexity of the class. In general it is clear that this class of chick lens structural proteins contains a number of different molecular species but estimates vary as to the number of different proteins involved, as well as their molecular weight. Differential centrifugation (Hoenders, 1965) and gel filtration (Truman, 1968) both indicated two major classes differing in molecular size. Using two dimensional polyacrylamide electrophoresis Zwaan (1968) found nine beta-crystallins, five of molecular weight 55,000, two of 60,000, one of 50,000 and one of 40,000. Truman et al. (1971) estimated by gel filtration that their molecular weights range from 16,000 to 59,000. Using high resolution techniques of polyacrylamide electrophoresis Zwaan (1968) could find nine components, Truman (1968) and Clayton (1969) found twelve. Bours (1971) with gel electrofocusing, attributed a minimum of ten bands to beta-crystallin components.

Arguing from indirect evidence including immunological arc patterns and studies of immunological cross reaction, Clayton and Truman (1967) proposed that beta-crystallins were a family of heteropolymers, each

molecule containing several subunits, some of which have a restricted range of electrophoretic mobility, whilst other members of similar or identical antigenicity are distributed with these, throughout the entire range of electrophoretic mobilty. The beta-crystallin precipitin line (the "long line") shows a number of spurs with homologous antisera, indicating partial immunological identity, probably reflecting the existance of polymers of different composition with at least one subunit in common (Clayton and Truman, 1967). When tested however with an anti-serum to amphibian lens (Xenopus laevis), a continuous precipitin arc is obtained, without spurs, indicating reaction with a determinant widely distributed in the beta-crystallins (Campbell et al. 1968; Clayton and Truman, 1974). Zwaan (1968) also suggested that a subunit structure could account for the variation in molecular size of the beta-crystallins. Using low resolution chromatographic techniques in dissociating conditions Rana and Maisel (1970) could detect beta-crystallin components made up from 4-6 subunits. Some subunits were common to all beta-crystallins, whilst others showing immunological reactions of non-identity had a non-uniform distribution. This evidence of a heteropolymer subunit structure is supported by the reduction in sedimentation coefficients (Clayton and Truman, 1967) and of molecular weight (Truman et al. 1971) in the presence of 8 M urea. More direct evidence comes from the work of Clayton (1969) who separated beta-crystallins by polyacrylamide electrophoresis then re-electrophoresed them in dissociating conditions. Her results indicated that the beta-crystallins were a family of heteropolymers whose mobilities are related to their subunit structure. Six major and five minor types of beta-crystallin subunit could be detected by this technique. Individual intact beta-crystallins consisted of several of these subunits, whose antigencity varies, only some of them showing species cross reaction.

The time and sequence of appearance of beta-crystallin in the chick lens have been repeatedly studied. The later results indicated that some

Table 3. Earliest demonstration of lens β-crystallin by various authors.

Anodal	Cathodal	Total	Reference	Technique
16 day embryo	100 hr. embryo		Rabaey (1962)	Immunoelectrophoresis
7 day embryo	10 day embryo		Zwaan (1963)	Immunoelectrophoresis
		56 hr. embryo	Zwaan and Ikeda (1968)	Immunofluoresence
54 hr. embryo	3½-4 day embryo		Truman et al.(1972a)	Osserman Test

beta-crystallins are synthesised much earlier in development than hitherto thought, with a time of appearance similar to that of alpha and delta-crystallin (Table 3). As pointed out by Clayton (1970), where authors have been able to distinguish between anodal and cathodal forms of beta-crystallin, there is agreement that the cathodal group appears first in development. The work of Genis-Galvez et al. (1968 b) also supports this conclusion. In the earliest age investigated by immuno-electrophoresis, the 15 day old embryo, only cathodal beta-crystallin could be detected. In contrast using the sensitive Ossesman technique, a modification of immuno-electrophoresis, Truman et al. (1972 a) were able to detect antigens of the cathodal beta-crystallin group partially identical with adult components as early as about 54 hours, whilst all the antigens occuring in the anodal group of beta-crystallins were present in the 3½-4 day old embryo. These differences in result partly reflect continual refinement of immunological techniques but also underline the fact that antibodies made against beta-crystallins are far more variable than alpha or delta-crystallin antibodies (Clayton, 1970). Although the relative proportions of beta-crystallin antigens in the adult and late embryonic lenses appear very different, Truman et al. (1972 a) attributed this to quantitative changes in the proportions of beta-crystallin rather than involving the synthesis of additional polypeptide chains after about 18 days of incubation. Postnatal development shows a progressive increase of anodal beta-crystallins (Genis-Galvez et al. 1968 b) and this class appears to be characteristic of the highly differentiated lens fibre cell. Thus the early annular pad, accordingly to these authors, contains virtually only cathodal beta-crystallins, whilst later on in development it contains both anodal and cathodal forms, the latter however predominate throughout life, in this region. In contrast wherever fibres are formed, anodal beta-crystallins are added.

The above summary outlines, in general, much of the information known about this exceptionally complex set of proteins, at the time this present investigation began. In an attempt to evaluate and add to this information, in order to throw further insight onto the differentiation of the lens fibre the following approaches have been made, in addition to those described for alpha-crystallin:

(1) The range of subunit molecular weights has been determined using a high resolution technique of urea-sodium dodecyl sulphate electrophoresis in polyacrylamide gels (Chapter 9).

(2) The subunit composition of intact crystallins isolated by gel electrofocusing have been compared to the antigenic patterns of these intact crystallins revealed by tests with antisera to beta-crystallin subunits. Some preliminary results are reported in Chapter 8.

(Unfortunately anti-sera directed against single subunits of alpha and delta-crystallin were not available.)

SECTION II THE STABILITY OF MESSENGER RNA.

INTRODUCTION

Implications of differential mRNA stability

There is increasing awareness that the regulation of protein synthesis is a key to understanding cell differentiation. The rate of synthesis of any given protein may be affected by (amongst other things) the amounts of available mRNA, which is in turn governed by its own rate of synthesis, controlled at the gene level, and the rate at which it decays. The rate of decay varies widely among individual mRNA species, for reasons as yet unkown. Although the stability of mRNA is thus a crucial factor in any model for the control of protein synthesis our knowledge of messenger turnover is indeed fragmentary.

If template molecules have a high decay rate then changes in transcriptional patterns will be rapidly registered in protein synthesis. With such unstable templates, continual synthesis is necessary with the advantage, however, that rapid changes in response to new environmental stimuli are possible. Thus in rapidly changing environments the need for adaptability to new conditions may well override the innate wastefulness of constant renewal.

There will be an obvious economy of energy and nutrients, however, in producing essential molecules on long-lived templates, particularly in cells not subject to major environmental changes. Whilst with templates that decay immediately and exponentially, synthesis of proteins can be controlled simply through cessation of template synthesis, with long-lived templates this control will not be achieved so readily. Now new patterns of protein synthesis will be determined by several factors including;

1) the kinetics of mRNA decay.
2) the size of preformed template pools.
3) the activities of degradative molecules such as nucleases and proteases.

In consequence there will be selective pressure on cells containing relatively stable mRNA's to develop more sophisticated levels of protein regulation than simply synthesis or non-synthesis of specific templates. Where the ratio of one particular messenger halflife compared to another, in the same cell, is markedly different, then several important consequences follow, as shown in the excellent article by Kafatos (1973).

Primarily the gene for a stable messenger needs to be transcribed much less often than that for an unstable messenger, to maintain the same level of protein synthesis. This is virtually a statement of the obvious, but Kafatos (1973) has shown by a simple calculation the extent to which a cell can devote a vast percentage of its total protein synthesis to a specific protein merely by synthesising a tiny percentage of specific messenger, provided certain assumptions are made. These are that mRNA synthesis is a zero-order process, i.e. independent of the amount of mRNA produced, and more importantly, that mRNA decays exponentially, i.e. that the decay reaction is dependent on the concentration of existing messenger. When the synthesis of mRNA is just equivalent to the amount decaying, the author has shown that the synthetic rates of any two messenger classes are inversely related to the ratio of mRNA halflives. The greater this ratio is, the lower the synthetic rate of the more stable mRNA needs to be. For example where one mRNA has a halflife 40 times greater than another, the rate of synthesis of the more stable mRNA needs to be only 2.4% of the total mRNA synthesis to maintain indefinitely 50% of overall protein synthesis. This is a strikingly economic rate of transcription for any highly specialised cell. Even where the range in mRNA halflife is not so marked, accumulation of the longer lived classes would be quickly obtained, on the basis of this calculation.

Secondly, constant production of a stable messenger will result in progressive acceleration of synthesis in the corresponding protein. This phenomena is of great interest since it means that dramatic shifts in the degree of cellular specialisation can take place in very short periods of time. Choosing realistic specifications and values derived from the literature, Kafatos (1973), used computer techniques to investigate a hypothetical system where the synthesis of a specific protein is coded for by a mRNA 40 times more stable than the average non-specific mRNA. He showed that to raise the synthesis of this specific protein from 0.1% of the total protein synthesis to 70% or 50%, in a period of 96 hours, requires only a constant mRNA synthetic rate of 10.4% or 4.8%, respectively, of the total mRNA synthesis. To achieve a stage where the specific protein synthesis reaches 20% of the total protein synthesis, after 96 hours, the constant rate of specific mRNA synthesis need only be 1.2% of the total mRNA synthesis. Even where variations in the stabilities of mRNA are not so marked, massive amounts of specific protein can still be made with comparatively low levels of specific mRNA synthesis. Where specific mRNA's are highly stable compared to the bulk of other mRNA's, remarkable changes of cellular protein content can be achieved by very low rates of constant specific mRNA synthesis. With the mRNA 5, 10, 40 or 100 times more stable than the non-specific mRNA, the author's calculations show that the rate of specific mRNA synthesis needed to achieve a 50% transitional specialisation in 96 hours, is only 29%, 17%, 4.8% or 2.0% of total mRNA synthesis respectively.

The author finally points out that differential mRNA stability can increase the flexibility of cellular response to developmental stimuli (such as hormones). Even where large numbers of genes have been stimulated to transcribed, if mRNA's are produced of diverse half-

lives, the expression of individual genes will vary widely, as judged by the synthesis of the corresponding protein over time. Where the bulk of the mRNA is stable there will be a considerable lag time before new transcriptional events override old, hence translational events can be programmed to occur long after the initial stimulus.

It might also be pointed out that long lived molecules are more likely to be available for transfer to progeny cells that those turning over rapidly. Provided mRNA is not destroyed during mitosis, then the longevity of the molecules will ensure a high degree of continuity between parent and daughter cells.

These theoretical considerations stress the importance of the stability of mRNA in cellular differentiation by both making possible considerable flexibility in developmental programming, and by providing a rapid and economic means of producing a high degree of protein specialisation within the cell. These conditions are not merely academic, however. Kafatos himself, working on the cocoonase zymogen cells of silk moths, has shown by indirect evidence from autoradiography and inhibition studies, the mRNA's of diverse stability can co-exist in the same cell (summarised in Kafatos, 1972, 1973). The main protein class produced by these cells, the zymogens, are made on remarkably stable mRNA, whose halflives (approx, 100 hours) are forty times greater than of non-specific mRNA within the same cell. Direct evidence of a long-lived messenger producing fibroin has been produced by pulse chase methods in the silk glands of Bombyx mori (Suzuki and Brown, 1972). Thus these highly differentiated cells specialise by progressively accumulating mRNA's specific to their function.

Concepts derived from the initial evidence of differential mRNA stability.

In general, RNA transcription and messenger translation are closely coupled events in prokaryotes and metabolic adaptability is

achieved by rapid turnover of mRNA. Messenger templates turnover in a few minutes, or within 2-5% of a cell generation time (Levinthal et al. 1962; Mangiarotti and Schlessinger, 1967; Adesnik and Levinthal, 1970). However differential stability may occur in bacteria, for example in the process of sporulation (Roses del Valle and Aronson, 1962) and in constitutive penicillinase mutants (Yudkin, 1966). If the unicellular algae Acetabularia is enucleated, the synthesis of several enzymes becomes affected at very different times, yet basic metabolic processes are not inhibited during the first few weeks after enucleation (reviewed by Keck, 1969). In such cases, the cells are committed to a stable synthetic function, so the use of stable mRNA is an obvious economy.

Similarly many of the examples of long-lived mRNA's in eukaryote cells are those of terminally differentiated cells such as blood platelets (Booyse and Rafelson, 1967), ~~erthyocytes~~ erythrocytes (de Bellis et al, 1964), feather keratin cells (Humphreys et al. 1964) and as discussed below, the lens (Reeder and Bell, 1965, 1967; Stewart and Papaconstantinou, 1967 a,b). In the majority of cases the nucleus has been inactivated or lost or cell division is no longer continuing. In these cells, subject to few environmental changes, such stability can be viewed as a device to prolong the lifetime of the cell after enucleation, and as a means of avoiding constant renewal of molecules. In liver cells, on the other hand, early results indicated that proteins are synthesised on comparatively unstable templates (Wilson and Hoagland, 1967). These experiments indicated a messenger lifetime of about 3.4 hours, or within 15-20% of a cell generation time. This is understandable if the need for high metabolic versatility overrides the demands of economy.

However the rates of degradation of the shortest lived procaryote mRNA's are still 1-2 orders of magnitude higher than those found in eukaryotes. Consequently, in eukaryotes utilising relative stable mRNA, it would be difficult to bring about short-term changes in protein

synthesis merely by controlling the rate of initiation of mRNA transcription.

Clearly, evidence that stable and unstable messengers can co-exist in the same cell is of great interest since it implies that a selective mechanism for the regulation of protein levels in the cell may exist. An unequivocal demonstration that differential mRNA stability is of regulatory significance would seem to demand evidence that two identified proteins formed from templates of quite different halflives, both co-exist and are synthesised in the same cell. Evidence of different halflives from experiments utilising complex tissues can be objected to on the grounds that the results reflect cellular heterogeneity rather than genuine differences of stability within a single cell. Furthermore, evidence must be offered of differential stability of actively translated messenger, since the stability of an inactive messenger may be brought about by very different mechanisms. Finally the inference of mRNA stability should be direct, i.e. based on metabolic studies of an identified and characterised messenger, rather than the synthesis of the corresponding protein.

Clarification is also needed of the relative roles of extrinsic factors such as nucleases and template pool sizes, and those of intrinsic factors such as secondary structure and mode and kinetics of decay, in any model of regulation through differential mRNA stability. Again, in an ideal experiment , the data should indicate whether a diversity of mRNA halflives exists within a single cell type, or whether regulation occurs through an all-or-none process e.g. the messengers halflives are similar but vary with, say, the cell cycle or metabolic activity of the cell. In other words regulation through differential turnover of messenger must be established as quantitative or qualitative, selective or general.

Recent results on the differential turnover of mRNA.

Whilst no experiments published to date satisfy all of the criteria listed above, recent results from a variety of systems imply that the stability of mRNA is significant as a factor in the regulation of protein synthesis. Some of the most recent results are discussed in detail in the next few sections. They are included to give an idea of the universality of differential mRNA stability, the variety of the techniques employed in reaching this concept and some idea of the significance of the concept for models of protein synthesis.

1) Stability of mRNAs in bacterial cells.

Perhaps the best example that stable and unstable messengers can co-exist in the same bacterial cell comes from the work of Marrs and Yanofsky (1971). They investigated the degradation of labelled mRNA in phage infected cells after the inhibition of RNA synthesis by rifampicin. In phage T7 an amber mutant is known for the gene which codes for the T7 polymerase. The only mRNAs produced by this phage, T7am193, are transcribed by the host polmyerase early after infection. Addition of rifampicin inhibits nearly all T7am193 synthesis in E.coli cells within 2.6 to 3.2 minutes. Consequently one can compare the rate of degradation of the phage mRNA with host mRNA, after the addition of the anti-biotic.

The authors compared T7mRNA and a product of the tryptophan operon, trpE mRNA. RNA samples were taken periodically and assayed for T7mRNA and trpE mRNA by hybridisation to the appropriate DNA. Their data reveals that the T7mRNA is relatively stable, with a half-life of approximately 6 - 20 minutes, whilst the trpE mRNA is labile, with a half-life of 80 seconds, in both phage infected and uninfected cells.

Since T7 infection has not altered the rate of degradation of trpE mRNA it has presumably not grossly altered the mRNA degradation mechanism, and yet the mRNA of T7 remains stable. The results suggest that some aspect of mRNA degradation is messenger specific, since the cell's mRNA degradation system, or systems, appear to recognise different mRNA species. Summers (1971) in an earlier study, found identical half-lives (6 - 20 minutes) for mRNAs of phage T7, although his findings suggested that both phage and host mRNAs were relatively stable. However, the elegant results of Marrs and Yanofsky (1971) appear to rule out a phage-mediated inactivation of the host's general mRNA degradation systems. The inhibitory effect of rifampicin on RNA synthesis has also been employed by Hirashima et al., (1973), but in this case the assay was indirect, being based on the effect of the antibiotic on the biosynthesis of well characterised proteins. They studied the stability of mRNAs for individual membrane proteins and cytoplasmic proteins of E. coli. At various time intervals after the addition of the antibiotic, the cells were pulse labelled with (^{3}H) arginine. The envelope and cytoplasmic fractions were prepared and separated by (SDS) polyacrylamide gel electrophoresis. The total radioactivity in various identified peaks, expressed as the percentage of a control (no antibiotic added), i.e. the rate of (^{3}H) arginine incorporation of the various protein peaks, could then be plotted against the treatment time. On average, the half-lives of mRNA for identified membrane proteins, calculated from the slope of this graph, were twice as long as those for cytoplasmic proteins. The average half-life for the two fractions is in fact 5.5 mins. and 2 mins., excluding a remarkably stable mRNA of a membrane lipo-protein which has a half-life of 11.5 mins. Clearly the mRNAs for the envelope proteins are more stable than those of the cytoplasmic proteins. However, the authors also investigated the inhibitory effects of several other antibiotics whose modes of action are to affect various aspects of translation of mRNA on the ribosome. As with rifampicin the authors found kasugamycin and puromycin were much more inhibitory to the overall synthesis of cytoplasmic

proteins than to that of the envelope proteins. Tetracycline and sparsomycin, on the other hand, inhibited the biosynthesis of envelope proteins much more than that of the cytoplasmic proteins. Finally, individual proteins of the membrane fraction showed differential sensitivity to the antibiotics. Together with the evidence of differential messenger stability for membrane and cytoplasmic proteins the authors concluded that the envelope proteins appear to be produced in a rather separate fashion from cytoplasmic proteins. Indeed, some of the membrane proteins may have their own specific synthetic systems. The authors speculate that structural differences in mRNAs may determine not only different stability of mRNAs but also different affinity of mRNA in binding to ribosomes. If the stability of the initiation complex is affected this may explain the differential sensitibity to antibiotics such as kasugamycin which are known to be inhibitors of initiation in protein synthesis.

One can speculate that, if, for example, translation restricts degradation by, say, making sequences of mRNA less susceptible to nuclease attack, then long-lived messenger may have a high efficiency of binding to ribosomes, and a stable initiation complex. The messenger stability could then arise merely from the fact that it is used more often, that is, the messenger is more often in a protected state of the ribosome, rather than its structure is more resistant to nucleolytic attack than that of a shorter-lived mRNA. Consequently both the structural integrity of the messenger and its affinity of binding to ribosomes may

play a part in determining the overall stability of a particular bacterial messenger.

The results of these recent experiments on messenger stability in E. coli can be summarised:

a) some aspect of mRNA degradation appears to be specific to the messenger

b) mRNAs for membrane proteins appear (on the average) about 2.5 times more stable than these for cytoplasmic proteins.

c) some membrane proteins may be produced by a separate synthetic system to that of cytoplasmic proteins.

The results imply a considerable level of sophistication in the mRNA degradation system and that at least two classes of mRNA stabilities can exist in a bacterial cell. Furthermore, all of the possible mechanisms discussed by Hirashima et al. (1973) whereby envelope proteins synthesis could be made on a separate specific system imply a considerable complexity in the overall regulation of membrane synthesis. These mechanisms include possible differences in the ribosomes themselves, or compartmentalisation of ribosomes, by membranes for example. Alternatively, the specificity may lie in various factors such as initiation factors, elongation and termination factors found on the ribosomes. Finally, as discussed the differential stability and affinity of mRNA to ribosomes may be involved. The major point of interest to note, however, is that already more sophisticated levels of protein regulation are implicated in bacterial membrane production than simply synthesis or non-synthesis of mRNA. Furthermore,

the two protein peaks which were most resistant to the antibiotics are both structural lipoproteins of the outer membranes: The mRNA of the smaller protein is extraordinarily stable with a half-life of 11.5 minutes. Such proteins are liable to be required continuously and stable messengers for such proteins will restrict the depletion of metabolites needed in constant renewal of cellular membranes. A similarly long-lived mRNA has been reported for an extra-cellular protease of Bacillus amyloliquefaciences. (Both et al. 1972).

Thus, the picture that emerges from these prokaryote researches suggests that messenger stability is not restricted to particular synthetic functions such as sporulation or phage production but rather that mRNA stability is also utilised in basic synthetic pathways, presumably as an economy of energy and nutrients. However, even the longest-lived bacterial $_{m}$RNA molecules exist for only a few minutes. Since the availability of this messenger regulates the synthesis of specific proteins, control of transcription and of the efficiency of transcription are liable to be the key regulatory points of genetic expression in bacteria.

2) Stability of cytoplasmic messengers in eukaryote cells.

Perhaps the most meaningful comparison to make to bacterial systems is to estimate the half-life of cytoplasmic mRNA in rapidly growing cells. Early studies indicated that in this situation the half-life of the mRNA is relatively short, about 3-4 hours, (Penman et al., 1963; Craig et al., 1971) compared to differentiated cell types such as reticulocytes, (Rifkind et al., 1964) lens, (Stewart and Papaconstantinou, 1967) or feather keratin cells,

(Humphreys et al., 1964), where mRNA has a half-life of several days. These estimates of the half-lives of mRNA in rapidly growing cells, whilst differing by one or two orders of magnitude from those for bacteria, nevertheless indicated that the lifetime of a mRNA molecule might be only 15-20% of a cell generation time, in these conditions, compared to 2-5% of a cell generation time in bacteria. This suggested that a roughly comparable rate of renewal of mRNA molecules per cell generation time occurred in both systems. However, recent technical improvements in the isolation of mRNAs have led to quite different conclusions about the stability of mRNAs in higher organisms.

The two major methods used to investigate metabolic turnover of mRNA in eukaryotes, according to Endo et al., (1971) has been:

1) the investigation of pre-labelled mRNA in polysomes after inhibition of RNA synthesis by actinomycin D,
2) measurement of changes in specific activity of mRNA in pulse labelling experiments where all species of RNA have previously been labelled to a nearly uniform specific activity.

The first method involves the use of an inhibitor, actinomycin D, with known secondary toxic effects (Revel et al., 1964, Soeiro and Amos, 1966). In particular this antibiotic is known to cause selective degradation of free poly-ribosomes in mouse liver, (Sarma et al., 1969). Consequently it appears that polysome breakdown in actinomycin may involve factors other than the decay of pre-existing mRNA. Perhaps more seriously, the decay of protein synthesis that follows the exposure of eukaryote cells to actinomycin D has been ascribed to the disappearance of mRNA, due to the degradation of messenger molecules in the period of inhibition.

This interpretation assumes that it is the availability of messenger molecules that is the rate limiting factor in protein synthesis under these conditions. The validity of this conclusion, that it is the decay of mRNA which is responsible for the decay of protein synthesis during inhibition of RNA synthesis by actinomycin has been strongly challenged (Singer and Penman, 1972), for the reasons discussed below.

The second method requires a precise and highly selective method for distinguishing radioactivity incorporated into mRNA from that incorporated into tRNA and rRNA. Since evaluation of the exact contributions of radioactivity from the different RNA species is extremely difficult wherever the isolation method does not exclude overlapping of different RNA regions, early pule-chase experiments may have given inaccurate estimates of messenger turnover.

Fortunately, new techniques for the isolation and purification mRNA allow pulse-chasing experiments to be carried out that do not involve inhibitors of RNA synthesis, and largely eliminate contribution to radioactivity incorporation by other contamination RNA species. Since these experiments appear to measure directly the kinetics of mRNA formation and decay, and since the estimates of messenger half-life obtained from them differ considerably from earlier results, the experiments require to be considered in some depth. These recent experiments have utilised the discovery that a high percentage of mRNA is mammalian cells contain sequences of polyadenylic acid (poly A), of about 200 nucleotides in length, found at the 3'-OH terminus of the molecule. Molecules containing such a segment can be isolated by virtue of its affinity for

polyuridylic acid (poly U) immobilised on a glass fibre filter (Sheldon et al., 1972) or oligo-deoxythmidylate (oligo-dT) immobilised on cellulose (Aviv and Leder, 1972).

In two illuminating papers (Singer and Penman, 1972: Singer and Penman, 1973) the authors have used both these techniques to investigate the metabolic turnover of mRNA in Hela cells. In the earlier paper they also showed that mRNA appears quite stable after the inhibition of RNA synthesis by actinomycin D. If, in fact, mRNA is disappearing after the addition of actinomycin, due to the degradation of pre-existing messenger, then the total number of polysomes in the cell should decline, but the sedimentation distribution of the polysome should remain similar. In fact polysomes extracted from Hela cells treated with actinomycin sediment slower than those from untreated cells, implying that the mRNA is now more lightly loaded with ribosomes. If, however sufficient cyclo-heximide is added to actinomycin treated cells to decrease the rate of ribosome movement along the message about 5-fold, then a significant recovery of normal-sized polysomes can be obtained.

The authors suggested actinomycin may decrease the rate of initiation of translation relative to the rate of elongation. Consequently, the ability to rebuild polysomes in the presence of cyclohexamide would be due to the new balance between this decreased initiation rate and the now dimished rate of elongation. The recovery of virtually a normal size range of polysomes several hours after the start of actinomycin treatment suggests that little of the mRNA has been degraded in this time.

The direct assay of messenger contents was attempted using poly-U filters. Most of the labelling of poly-A containing RNA of Hela cells selected through this filter technique is inhibited by cordycepin, an adenosine analogue, which has been shown to be a specific inhibitor of the labelling of cytoplasmic $_{m}$RNA, (Penman et al.,1970). The poly-A containing RNA is found to sediment with polyribosomes, to be released by EDTA and to have the characteristic sedimentation of MRNA. On these criteria at least, it behaves as functioning mRNA. The RNA from cells treated with actinomycin which binds to poly-U filters appears identical in size and purity for various times of treatment. Furthermore, even after 5 hours in actinomycin there is little decline in the content of RNA containing poly-A. Following this basic finding that mRNA in exponentially growing Hela cells appears to have a half-life appreciatively longer than previous estimates, the authors made more detailed measurements (Singer and Penman, 1973).

In this set of experiments, the poly-A containing messenger fraction was isolated by virtue of its affinity for oligo-dT cellulose. This afforded the reproducibility and precision in the experiments that is not available with the poly-U glass fibre technique. The evidence by which the poly-A containing material can be considered to be functional mRNA is given above. Two methods were used to measure the $_{m}$RNA lifetime. One method measures decay in the poly-A containing fraction over several days after a brief period of pulse-

labelling. Alternatively, the continuous labelling of mRNA can be compared both at the beginning of a labelling period and after the stady state ratio of mRNA label to rRNA label has been achieved. In fact, the authors derive the messenger half-life as a ratio of mRNA label to that in rRNA, which is known to be stable for at least 54 hours. If cells are incubated in the presence of $^{32}PO_4$ for 6 days then all species of RNA will be labelled to nearly uniform specific activity. By comparing the material selected byoligo-dT affinity to that of the rRNA that remains unbound, measurements made in these steady state conditions reveal that the amount of mRNA is about 5.2% of the total rRNA or 18% of the 18s rRNA in the cell. A direct determination of messenger half-life can then be made by labelling cells briefly with uridine and following the label in mRNA and rRNA. If the ratio of mRNA to 18s rRNA labelling is plotted, the labelling kinetics are complex, but can be fitted by assuming two populations of mRNA, one with a half-life of 6 - 7 hours, the other a long lived component with a half-life of 24 hours. By labelling mRNA first with (^{14}C) uridine and then with (^{3}H) uridine, 19 hours later, one can compare two populations of mRNA, one old (^{14}C) labelled and one new (^{3}H) labelled. The decay of both populations is then plotted relative to their respective 18s rRNA on a logarithmic scale. The two mRNA components with different decay times were again observed, but the old mRNA has only a 24 hour half-life, whilst the new (^{3}H) labelled mRNA shows biphasic decay, with a major 6-hour component. About 60% of the mRNA decays rapidly and about 40% slowly in the newly formed message. Measurements made in steady state conditions

reveal that 33% of total cellular mRNA comprises the rapidly decaying species, and 67% possesses a longer lifetime.

The lifetime of mRNA in the presence of actinomycin, however, is significantly shorter. The decay of both old and new RNA can still be observed in these conditions, but the more rapidly decaying components displays a half-life of about 4.5 hours, whereas the long lived component has a half-life of about 12 hours. These results are achieved simply by labelling the cells as before, then adding actinomycin D and removing portions from the cell culture at various times. The ratio of (^{14}C) labelled mRNA (old) to (^{3}H) labelled mRNA (new) can then be plotted against time. Old mRNA again decays more slowly than the newly labelled material. The shorter decay times suggest that mRNA may be degraded more rapidly in the presence of actinomycin, but the half lives are still sufficiently long to emphasise that it is not the supply of mRNA that is rate limiting in protein synthesis during ~~initiation~~ inhibition of RNA synthesis by actinomycin.

The data shows that the old mRNA and newly labelled mRNA behave differently. This is direct evidence of the presence of two populations of mRNA within a single cell type, and suggests that more than one mechanism may exist to degrade mRNA in eukaryote cells, a situationsimilar to that in phage infected bacteria, (Marrs and Yanofsky, 1971).

These basic findings have been extended further by the same laboratory in their study of the effect of actinomycin D on the control of translation in Hela cells (Goldstein and Penman, 1973.). The authors have re-examined the decay of protein synthesis in actinomycin treated Hela cells in cell culture conditions that avoid excessive shearing of cells. In these conditions when

actinomycin D is present, the half-life of protein synthesis, normally about 6 hours, can be reduced to only 2 hours by simply increasing the temperature of cell incubation to 42°C. There is no corresponding increase in the decay of the mRNA. At 42°C. approximately 83% of the total mRNA remains, compared to the 37°C. control rate. This remarkable decoupling of protein and mRNA synthetic rates again suggests that the decline in protein synthesis in actinomycin treated cells is not primarily caused by decay of the available mRNA. The mRNA remains functional at this temperature, since the reduced polyribosome profile in actinomycin can again be restored to normal distribution and amount by the addition of cycloheximide, which slows translation relative to initiation. The translation rate, which is independent of the rate of initiation is virtually unaltered at 41°C, as compared to 37°C, even though protein synthesis at this temperature is severely reduced.

The authors suggest that actinomycin D interferes with the normal replacement of a factor necessary for initiation of protein synthesis. The sensitivity of this postulated factor to actinomycin D appears to be less than that of ribosomal RNA but considerably greater than that of tRNA,5s and the bulk of mRNA. on the basis of dose response studies. A factor which can be produced during cycloheximide inhibition, promotes protein synthesis at elevated temperatures apparently by stimulating the initiation of translation (McCormick and Penman, 1969). This factor is also actinomycin sensitive and is presumed to contain RNA. Prior incubation of cells in cycloheximide prevents, at least partially, the sharp decrease in protein synthesis that occurs at 42°C. The induction of the

protective effect of cycloheximide is inhibited by the same amount of actinomycin D as is required to decouple protein and RNA synthesis, hence the authors conclude that a similar or identical RNA-containing factor which promotes the association of ribosomes with mRNA can no longer be supplied in the presence of the drug.

However before the implications these results have on our concepts of mRNA stability are discussed the results simultaneously elucidated by experiments with mouse L cells (Greenberg 1972, Perry and Kelley, 1973) must also be taken into consideration.

Greenberg (1972) measured the turnover of poly(A) containing messenger in exponentially growing mouse L cells by studying the kinetics of polyribosomal RNA as it approaches steady state-uridine labelling. He showed that the RNA was labelled from precursor pools of constant specific activity by following the time course of rRNA labelling. Such labelling follows a theoretically predictable course for a stable component, if one assumes that RNA appears in the polyribosomes one hour after the beginning of labelling. The derivation of the equation for the curve is discussed in this paper (Greenberg, 1972) and in further detail in the appendix of a second paper (Perry and Kelley, 1973). Having shown that the specific activity of the pools of radio-active precursors remains constant over the whole of the labelling period, he then measured the kinetics of labelling of poly-A containing mRNA. The labelling of such RNA could be measured at various times by binding polyribosomal RNA to 'Millipore' filters under conditions specific for poly-A adsorption (Lee et al., 1971). The steady state curve so obtained could then be compared with theoretical curves for various half-live values. The 'best fit' approximation to a theoretical curve, as judged by visual inspection was obtained with a half-life value of 10 hours, and a lag of 0.25

0.25 hours in the time of appearance of the poly-A containing mRNA on the polyribosomes. The mean life-time of such a messenger would be virtually equal to the generation time of the exponentially growing L cells. The results indicate again that the lifetime of mRNA in mammalian cells is very long, both Hela cells and mouse L cells apparently include a population of poly(A)-containing mRNA that turns over about once per cell generation. However the equation derived by Greenberg (1972) to express the kinetics of labelling polyribosomal RNA in an exponentially growing population of cells is derived on the assumption that the mRNA molecules decay in a stochastic manner. Thus decay kinetics are represented by a first order process in which the turnover rate constant of the messenger is independent of time, implying that old and new mRNAs have equal probabilities of veing destroyed. Consequently since his data fits adequately a theore tical curve derived with a single value for the half-life of messenger, the implication is that in mouse L cells there do not appear to exist multiple components in the poly(A) containing mRNA with distinctive differences in mean half-life times. Such a homogeneity with respect to half-life values of mRNA would appear different from that revealed in Hela cells where pulse chase techniques indicated at least two populations of mRNA with distinctive half-lives. existed. This discrepancy is solved to a certain extant by further observations of Greenberg's colleagues (Perry and Kelley; 1973) on messenger RNA turnover in mouse L cells. They compared the turnover of poly(A) containing mRNA with that of histone mRNA, a message believed to lack poly(A) (Adesnik and Darnell, 1972; Greenberg and Perry, 1972a, and which appeared to turn over more rapidly than other types of mRNA (Craig et al., 1971). This suggested that poly(A) sequences may have a role in determining mRNA stability .

The assay for poly(A) (+) mRNA utilised the poly(U) glass fibre techniques (Sheldon et al., 1972). Only functional histone mRNA turnover, that is the histone mRNA found on polyribosomes was measured. Histone mRNA disappears from polyribosomes soon after DNA synthesis is inhibited by cytosine arabinoside (aCyt) (Borun et al., 1967). The fractionation on 6% polyacrylamide gels of control RNA can be compared to that of RNA from cells treated with aCyt one hour before harvesting, and the difference assumed to represent histone mRNA activity. The authors claim this method allows unambiguous identification and that such measurements of histone mRNA labelling are accurate to within about 5%. Thus they define the histone mRNA as an aCyt-sensitive RNA component of about 130,000 to 150,000 molecular weight as revealed by electrophoresis. The authors employ exactly the same technique as derived by Greenberg (1972). They show that the stable species such as tRNA follow the theoretically derived curve, emphasising that the specific activity of the radioactive precusor remains constant over the entire experimental period. By substituting various half-life values in the equation one can generate a family of curves, and the poly-A(+) mRNA again displayed a mean lifetime of about 15 hours in cells growing 37°C with a doubling time of 13.5 hours. Simililarly in cells growing at 30°C with a generation time of 41 hours, the mean lifetime of mRNA was estimated to be about 42 hours. The mean lifetime of mRNA per cell generation thus appears to remain constant. The results are in good agreement with Greenberg (1972), and the closeness of the fit to a theoretical curve derived on the assumption that the mRNA molecules decay stochastically again suggests that the bulk of poly A(+) mRNA turnover with first order (stochastic) kinetics.

However the labelling data for histone mRNA does not follow any of the family of curves generated by the theoretical equation. One can modify this equation by introducing a function in which the probability of decay increase linearly with time, and the kinetic data of the histone mRNA does give a good fit to such a sigmoidal aging curve. A further modification to the equation accounts for the possibility that the mRNA molecules may have a fixed life-time, decaying at some specified moment, perhaps at some particular stage of the cell cycle. The kinetics data for the histone mRNA shows an even better fit to such a curve derived on the assumption that the mRNAs molecules have a fixed uniform limetime, suggesting histone mRNAs remain functional for a fixed time after assimilation into polyribosome. The authors find the concept of a fixed lifetime is especially attractive since histone mRNA is believed to function only during the DNA synthetic (S) phase, (Gallwitz and Mueller,1969), and their value for the fixed lifetime of histone mRNA (11 - 8 hours) compares reasonably well with the value of 8.5 - 9 hours they find for the duration of the S phase. They favour a model to explain the ordered process of histone mRNA turnover, wherein the histone messages are destroyed (or rendered unusable) in a "sensitive period" at the end of the S phase and are synthesised de novo for each succeeding cell generation. The value derived for the mean lifetime of the histone messenger (11.3 hours) is considerably greater than their earlier estimate (1 hour) based on the decay of polyribosomes in actinomycin. (Craig et al., 1971). This short lifetime may reflect the cessation of DNA synthesis which occurs as a secondary toxic effect of actinomycin D. These results again emphasise the need to correlate data obtained from the use of this antibiotic, wherever possible with a

kinetic analysis that avoids conditions of inhibition.

Apart from showing differences in the mode of decay of poly(A) containing mRNA and histone mRNA, the results also shed light on the possible roles of poly(A) in messenger turnover. As mentioned earlier, the poly(A) sequence is absent in the histone mRNA, which appeared to turn over far more rapidly than poly A containing mRNA. The poly(A)- sequence is also known to be particularly resistant to ribonuclease digestion (Kates, 1970) and taken together the data suggested poly(A) may have a role in determining mRNA stability by considerably delaying hydrolysis of nucleotides from the 3' - OH end of the molecule. However the observation that poly A containing mRNA decays stochastically, that is old and new mRNAs have equal probabilities of being destroyed, whilst the poly-A segment is continually shortened over the course of the lifetime of the mRNA molecule (Greenberg and Perry, 1972; Sheiness and Darnell, 1973), suggests that mRNA stability does not depend on the absolute length of the poly(A) segment. During the time of synthesis of the histone mRNA it appears no less labile than poly(A) containing mRNA; but the authors speculate that the poly A sequence may be involved in protecting mRNA during the sensitive period in the cell cycle when the histone messengers are rendered unusable. It has also been suggested that the absolute length of the poly A sequence could indicate the number of times a particular messenger has been translated. In this ticker-tape model (Sussman, 1970) an adenosine nucleotide is presumed to be removed from the sequence say, every n^{th} time a ribosome traverses a mRNA molecule until after a certain number of translations (a maximum of 200 since there are approximately 200 nucleotides in the poly(A) sequence) the molecule is virtually devoid of poly A and becomes highly susceptible to nuclease attack.

Again if the kinetics of decay are random for the poly A containing mRNA, then this sort of model is ruled out. The simplest form of the ticketing model in which one adenosine nucleotide is removed per translation was already unlikely in the light of the calculation that a typical message is translated 16 times per minute in eukaryote systems, where mRNA exists for several hours (Kafatos 1972). (For example one can calculate that a messenger being translated for 10 hours will be read 9,900 times, consequently the 200 long nucleotide sequence of poly A could only be clipped of one nucleotide once every 48 - 49th reading). More recent evidence on the relationship between poly(A) sequences and mRNA stability is discussed later (see Discussion).

In the L cell experiments discussed here (Perry and Kelley,1973) there is no evidence of mRNA components with distinctive decay times, as revealed in Hela cells by the pulse chase method of Singer and Penman (1973). Possibly the poly-U glass fibre technique is not sufficiently accurate or reproducible to reveal multiple lifetimes. However the authors cite unpublished results (Bard and Perry) on which a similar pulse chase technique of labelling 'old' and 'new' messengers (Singer and Penman, 1973) revealed more than one mean lifetime for poly A containing mRNA. However there still appear to be differences in the two cell types. In Hela cells, 33% of total cellular mRNA is composed of the shorter lived component, with 67% as the more stable species. At least 75% of the poly(A) containing mRNA in L cells appears to consist of long-lived components, However other differences in result are more difficult to explain.

The closeness of the decay curve of mRNA to that of a theoretical curve that assumes stochastic decay is strong evidence that old and new messengers are broken down with equal probability. In a short

pulse label, rapidly synthesised message would be expected to predominate, whilst in a steady state label, all classes should be labelled fully. If one separates by electrophoresis the poly(A)-containing mRNAs from both pulse label and steady state label experiments there appears no striking differences in the size classes of the mRNAs from mouse L cells (Greenberg and Perry, 1972b). However the double-label pulse chase technique reveals a significant difference in size of messengers in Hela cells (Singer and Penman, 1973), the newly synthesised messenger appearing larger than the old. (The authors also claim uncited evidence of a similar nature has been discovered in mouse cells). The presence of actinomycin D does not affect this result. The difference in size may reflect a post-transcriptional reduction in size, dependent on the time spent in the cytoplasm, since poly(A) sequences are known to be continually shortened over the lifetime of the mRNA molecule (Sheiness and Darnell, 1973). Alternatively it may represent a real difference in size of other nucleotide sequences at the time of synthesis, which would suggest that there may be some correlation between overall size and mRNA stability. At the moment it appears impossible to distinguish between these two alternatives but it should be noted that there is evidence against the concept of a correlation between mRNA stability and the size of the protein it eventually codes (Kafatos, 1972).

Both the difficulties mentioned here, of recording stochastic decay with the observation of two distinct messenger lifetimes, and conflicting evidence on the size of the newly synthesised messenger as compared to old, may merely reflect purely technical difficulties. For example Singer and Penman (1973) claim that only the oligo(dT)-cellulose technique for selecting poly(A)-containing RNA is sufficiently accurate and reproducible to give the required experimental precision.

But even with this technique they point out that very short lived components would not be detected because of the long time period before a chase becomes effective. (The nature of eukaryotic mRNA decay is considered further in the Discussion section, in the light of very recent evidence). Of crucial importance is the nature of the decay of the mRNA component which does not turn over with the bulk of the poly A containing mRNA in mouse L cells (Bard and Perry, cited in Perry and Kelley, 1973). This would be of great interest if the kinetic data suggest both populations of poly(A)-containing mRNA decay in a stochastic manner. For this would suggest that whilst the degradation of poly(A)-containing mRNA is caused by a series of random events, some difference in the populations of mRNA obtain so that the time in which a particular population decays is considerably different from that of another. One obvious factor that could provide an intrinsic difference in susceptibility to random ribonuclease attack is the secondary structure of the mRNA molecule. The idea that structural differences in mRNA are of key importance in explaining messenger stability has been mooted in investigations of bacterial systems (Marrs and Yanofsky, 1971; Hirashima et al. 1973). Even if the short lived components were shown to not turn over stochastically, i.e. the mode of decay was ordered, in the manner suggested for histone mRNA, (Perry and Kelley, 1973), considerations of secondary structure in determining mRNA stability, would not be out of place. This concept is considered further in a later section (see Discussion).

CONCLUSIONS

As argued earlier there will be an obvious economy of energy and nutrients, for example, to a primitive bacterial cell, if essential molecules are produced on long-lived templates. Recent evidence indicates that bacterial cells utilise mRNAs of differing stability, although even the longest lived messenger exists for only a fraction of the cell generation time. In eukaryotes the mRNA appears much longer lived than previously thought. Much of the bulk mRNA appears to turnover once per cell generation. This is of obvious economic advantage to the cell, whilst mRNA can be passed to progeny cells even though polyribosomes are mainly disagggregated during mitosis. This follows from the kinetic analysis of mRNA turnover in mouse L cells which revealed no marked decrease in the stability of poly(A) containing mRNA following its inactivation during mitosis (Schochetman and Perry, 1972a). For the bulk of the mRNA the mean lifetime is approximately equal to the cell generation time. By statistical definition, any mRNA molecule has a 63% probability of decaying within an interval equal to the mean lifetime (Perry and Kelly, 1973). Consequently a considerable number of molecules are still available for transfer to progeny cells. This previously translated mRNA appears to be retained in such a form as to allow a ready resumption of protein synthesis immediately after mitosis. It is highly likely that the mRNA is retained in the cytoplasm as free ribonucleo-protein and as mRNP-monoribosome complexes (Schochetman and Perry, 1972a). Thus a substantial degree of economy of metabolites and a high degree of continuity between parent and progeny cells is possible because of the longevity of the bulk of the mRNA. However the possession of a relatively stable population of mRNA means that the short term regulation

of protein synthesis will not be brought about merely by changes in transcription patterns since the total amount of mRNA will vary only very slowly in eukaryote cells.

The use of actinomycin D to investigate metabolic turnover of mRNA in eukaryote cells, long considered unsatisfactory because of toxic side effects, must be viewed with caution, in the light of the demonstration that it is not the supply of mRNA that is rate-limiting in protein synthesis during the inhibition period (Singer and Penman, 1972). Actinomycin D probably interferes with the normal replacement of a factor necessary for the initiation of protein synthesis (Goldstein and Penman, 1973). However the use of the antibiotic may be justified in cases where a change is expected to occur from unstable to stable messenger. This follows if new mRNA synthesis is effectively inhibited, since during a long period of incubation with actinomycin, very short lived messengers will decay, regardless of the effect of the drug on initiation events in protein synthesis. Because of these complications at the ribosome level however, it may no longer be valid to claim accuracy in estimates of mRNA half life derived indirectly from the synthetic pattern of the corresponding protein.

Finally, it should be noted that there is considerable confusion as to whether the mode of decay for mRNA is stochastic or ordered, or whether both types of decay can be found. There is also great ignorance of the manner in which eukaryote mRNAs are degraded by nucleases. Knowledge of the nature of mRNA degradation might well shed light on the molecular events that determine the mean life of individual mRNA species.

EARLY STUDIES ON MESSENGER STABILITY IN THE LENS

Scott and Bell (1964) noted that following the inhibition of RNA

TABLE 4.

Evidence from actinomycin D studies that chick lenses contain mRNA of different stabilities.

Chick Age	Reference
5-day old embryo	Clayton 1970, Clayton et al. 1972
6-day old embryo	Craig and Piatigorsky, 1973
8-day old embryo	Clayton 1970, Clayton et al. 1972
12-day old embryo	Reeder and Bell, 1965, 1967
14-day old embryo	Scott and Bell, 1965
1-day old post hatch	Clayton 1970, Clayton et al. 1972

N.B. Yoshida and Katoh (1971, 1972) claimed that the synthesis of all crystallins were stable in the 1 day post hatch chick.

synthesis by actinomycin D the level of protein synthesis soon dropped in the early developing lens. In older lenses this sensitivity of protein synthesis to actinomycin D diminished. This persistence of protein synthesis was ascribed to the presence of stable mRNA. Other workers reported similar conclusions for bovine lenses (e.g. Spector and Kinoshita, 1965, Stewart and Papaconstantinou, 1967a, b.). Numerous reports employing actinomycin D have indicated that embryonic chick lenses of various ages contain mRNA with different stabilities (Table 4). Much of this evidence has been reviewed in full by Clayton(1970) so it might be more valuable to discuss here, instead, the general experimental approaches made in these initial studies.

a) Use of Actinomycin D

Generally the early investigations on messenger stability used an indirect method, following the decay of protein synthesis that followed the exposure of the lens cells to actinomycin D, and ascribing this decay to the disappearance of mRNA, due to the degradation of template molecules in the period of inhibition, (Scott and Bell, (1965) had additional evidence however, of differential mRNA stability from the biphasic decay of chick lens polysomes). This premise may not be correct, given the evidence, cited above, that actinomycin D seriously reduces the initiation rate of protein synthesis, and that eukaryote mRNAs may have appreciably longer half lives than previously thought.

As argued above, where inhibition of RNA synthesis is virtually total, then actinomycin D can still give insight of changes in messenger stability, where long periods of incubation are used to offset the longevity of mRNA halflife. It may not however be meaningful to try and derive exact mRNA halflives through the use of this antibiotic. Unfortunately long periods of RNA inhibition increase the likelihood of

actinomycin D producing unwanted side-effects, including the non-physiological breakdown of rapidly-labelled RNA (Rovera et al. 1970, Scholtissek, 1972).

Other side-effects may occur that pertain directly to the lens. Thus the stimulation of amino-acid incorporation into protein in adult bovine fibres by actinomycin D is dependent on the presence of epithelial cells, (Stewart and Papaconstantinou, 1967a, b.). This effect may be artefactual if the transport system of the epithelium is affected by actinomycin D, (Clayton, 1970). Few authors have even considered whether actinomycin D differentially affects the rate of degradation of specific mRNAs. However chick crystallins have been shown to be stable in the presence of the antibiotic, (Craig and Piatigorsky, 1973), so that its effect does not result from a faster degradation of specific proteins. Slight stimulation of specific protein synthesis may be only apparent, since there is decreased incorporation of label into all affected proteins.

The effect of actinomycin D on amino-acid incorporation into whole lens soluble protein is also very time-dependent (Reeder and Bell, 1967, Truman, Clayton and Hunter, in preparation). Many of the initial experiments utilised very short periods of incubation with actinomycin D, so that maximum inhibitory effects may not have been detected. This criticism can be levelled at several investigations, e.g. Stewart and Papaconstantinou, 1967a; Clayton, 1970; Craig and Piatigorsky, 1973. On the other hand some workers have employed pre-incubation periods with actinomycin D of 24 hours (Zigman and Lerman, 1968) when there is evidence that eukaryote cells may recover from its effects after 14-17 hours after a single administration of the drug (Schwartz et al. 1965), suggesting that breakdown of the antibiotic does occur with time.

Clearly with these complications introduced by the use of actinomycin D, accurate data concerning the stability of lens mRNA during ontogeny and differentiation will have to depend ultimately on direct methods of pulse-labeling and extracting defined mRNA species. To this end preliminary attempts have been made in this laboratory to isolate different classes of polysomes by virtue of the antigenic specificity of the nascent polypeptide chains being translated, which contain the mRNA specific for that polypeptide against which the antibody is directed (Clayton et al. 1970; Clayton, 1970; Clayton et al. 1972). Eventually it should be possible by virtue of this immunological sampling to compare the stability of specific mRNAs which code for particular crystallin subunits under different conditions of growth and differentiation. Resort has had to be made however, in this investigation, to the use of actinomycin D as a RNA inhibitor, although knowledge of the difficulties and defects inherent in this approach means any subsequent results obtained are indicative rather than absolute.

b) Resolution

Most of the early published work on messenger stability in the lens, employing actinomycin D, has been made on crystallin heteropolymer aggregates rather than subunits. This applies both to bovine lens (Stewart and Papaconstantinou, 1967a, b.) and chick lens (Yoshida and Katoh, 1971, 1972, and Katoh and Yoshida, 1973). In consequence such aggregate values of stability will be biased towards the mean, whilst the behaviour of minor components that differ markedly from the average, might well be overlooked.

In some cases, even where dissociating conditions have been used the subsequent resolution of the soluble proteins has been so poor that the major bands portrayed must represent not single subunits but

unresolved complexes, so that only the most general of conclusions can be drawn. This is particularly true of the work of Reeder and Bell (1967) who could only resolve three major bands by urea-polyacrylamide electrophoresis, representing 83% of amino-acid incorporation. Two of these bands together accounted for 95% of the label in the body of the lens. None of these bands were identified with respect to crystallin class.

Evidence has already been presented above indicating that crystallin aggregates consist of several subunits, whose subunit composition changes during ontogeny. This means it is imperative to investigate the stability of mRNA for individual identified subunits, employing dissociating conditions and high resolution methods of protein analysis. Such an attempt is reported in Chapter 14.

SECTION III

RATIONALE FOR INVESTIGATING mRNA-ASSOCIATED PROTEINS

The evidence indicating that in a number of differentiated cell types very stable mRNAs exist, coding for proteins characteristic of the differentiated state, has been described in the previous section. In many cases as development proceeds the major proteins, typical of the fully differentiated tissue, are coded for by highly stable mRNA species, where previously they were made on relatively unstable templates. The mechanism of stabilisation appears to be highly specific for particular types of mRNA since other proteins may still be produced on short lived templates. Since cytoplasmic mRNA exists in the form of a ribonucleoprotein complex (see below) two main lines of investigation appeared possible in any study for factors involved in messenger stability. The structure of mRNA itself could be investigated or the proteins associated with mRNA species.

Ideally one would like to analyse directly the structure of an mRNA molecule coding for a protein typical of the differentiated tissue, to determine any modifications that take place during the transition period from instability to stability, preferably in a system where the levels of ribonuclease remain constant. This type of approach would require considerable expertise in sequencing mRNA and a highly characterised developmental system, conditions which precluded me from using the chick lens for investigations of this type. However in other developing systems this type of analysis now appears much more feasible, e.g. in myoblast differentiation (Buckingham et al 1974) or in enucleated reticulocytes (Williamson et al. 1974).

The second possibility, of investigating the proteins attached to mRNAs, appeared more realistic. At the time of commencement of the

work no functions had been ascribed to these proteins and there was a possibility that the ribo-nucleoproteins arose through non-specific RNA-protein associations in cytoplasmic extracts of eukaryote cells. When cells are lysed at low ionic strengths and polysomes isolated by methods employing detergents, the adsorption of extraneous proteins to RNA is a serious problem (Olsnes, 1970, 1971 a, b.). Obviously the possibility of the formation of complexes between RNA and cytoplasmic proteins through fortuitous binding brought into question the reality and significance of the mRNA protein complexes that had been described, but there were attractive initial reasons for investigating the biological function of the mRNA bound proteins. If proteins are specifically bound to mRNA, it is easy to visualise how mRNP complexes could be allosterically altered by cytoplasmic factors. The variability in type and degree of binding of the proteins to the mRNA might then determine the activation or inactivation of the mRNP complex. This type of rationale which has been consistently advocated by Klaus Scherrer and his co-workers, has been summarised recently (Gander et al. 1973):

"Proteins are not only susceptible to changes in their tertiary structure under the influence of ionic conditions, allosteric effectors or by chemical alteration (S-S bridges, phosphorylation); their much more complex biochemical structure also confers upon them a large variability in intrinsic stability. Therefore stability and activity of mRNA can certainly be governed much better by the intrinsic stability of associated proteins rather than by the secondary structure of the mRNA itself."

In this type of scheme the stability of the messenger is envisaged as an intrinsic property of the mRNP complex, and a function of the interplay of equilibrium constants controlling the mRNA-protein associations (Spohr et al. 1970). The life span of any particular

mRNA molecule in this model will be determined by the size and nature of the nucleotide sequences left exposed during dissociation, as well as by the length of time nucleases can act on the non-protected regions. A range of messenger RNA species with different half-lives can then be generated if the dissociation constants can be influenced by subtle changes in ionic conditions or allosteric modifications induced by cytoplasmic factors.

These kinds of considerations although based mainly on theoretical arguments provided an attractive model for the regulation of messenger stability. Therefore it was of interest to determine whether the polysomal mRNA species, in a cell producing many proteins, such as the chick lens, were associated with the same or different mRNA-binding proteins. When this investigation began it was not known whether the proteins associated with a full spectrum of mRNAs were identical, similar or unrelated. It also seemed ultimately possible to compare messenger classes with distinctive differences in stability and determine whether they were associated with different sets or quantities of the mRNA-binding proteins.

The characterisation of the nature of mRNP complexes and evaluation of their role in development remains in its infancy but an excellent and comprehensive review on the protein moieties of mRNPs has been recently produced (Williamson, 1973). Rather than retread this ground needlessly and since controversy exists in some cases as to whether the proteins are merely fortuitously bound contaminants, it seems of more obvious value to consider the evidence reflecting on the biological reality of the particles. The most recent evidence suggests that such RNA-protein complexes are not merely artefacts of the isolation procedure used in obtaining mRNP.

Whilst convincing evidence has accumulated recently that the mRNA of eukaryotes is present in the cytoplasm as a ribonucleoprotein (see the review by Ovchinnikov and Spirin, 1970), there is considerable controversy as to whether the proteins are non-specifically bound contaminants. One of the most commonly used methods of obtaining polysomal mRNP is to treat purified polysomes with the chelating agent EDTA, in low salt concentrations. Under these conditions the ribosomes split into sub-units and the mRNA so released appears to be complexed with an approximately equal amount of protein (Burny et al. 1970). Furthermore spontaneous non-specific binding of protein to several RNAs have been shown to occur at low salt concentrations in various eukaryote cell homogenates (Baltimore and Huang, 1970).

When reticulocyte ribosomes were dissociated by puromycin in high concentrations of salt, the ribosomal sub-units were obtained functionally intact (Blobel, 1971), in contrast to EDTA dissociation which inactivated both ribosomal sub-units. The mRNA released by the puromycin-high salt method was bound to two proteins with molecular weights of 52,000 and 78,000 (Blobel, 1972). These proteins remained bound to rabbit globin mRNA at 500 mM KCL in the absence of magnesium, yet these salt conditions produced complete dissociation of a few distinct ribosomal proteins from both ribosomal sub-units. The fact that only a minor number of proteins were detected in the mRNA-protein complex, and the resistance of this complex to dissociation by high salt concentrations as stressed by the author, points to a specific interaction between the mRNA and the protein rather than to non-specific adsorption of protein onto RNA.

The same procedure has been employed to obtain mRNP fractions from chick embryo cells, and again two distinct proteins (molecular weights

78,500 and 48,400) were found, associated with a spectrum of mRNAs (Bryan and Hayashi, 1973). The proteins involved are thus not a specific property of globin mRNA, and the finding that two proteins are common to and bound to several diverse species of mRNA again suggests that the mRNPs do not result from the non-specific association of proteins with RNA. Blobel (1973) has furthermore shown that highly heterogeneous cell types such as rat hepatocytes and mouse L cells have two proteins associated with their polysomal mRNA which are of identical molecular weight to those attached to rabbit globin mRNA. Thus the association of protein with mRNA in salt-resistant complexes may well be a feature of many eukaryotic templates. Independent and direct corroboration for his results with globin mRNA have been obtained by other experimenters working on cytoplasmic non-polysomal globin mRNP. Recently RNPs containing globin mRNA have been isolated from the post-ribosomal supernatant of rabbit reticulocytes (Jacobs-Lorena and Baglioni, 1972); this RNA is enriched substantially in alpha globin messenger activity, as determined by translation in a heterologous cell free system, and probably reflecting the relative amounts of free alpha and beta Hb-mRNA in these cells. The RNP contains the same two proteins as the polysomal mRNA, when sedimented through sucrose gradients (Gross and Baglioni cited in Gross et al. 1973). This tenacious binding of proteins to polysomal and cytoplasmic non-polysomal mRNA suggests that the RNP particles are of biological significance rather than artefacts formed during the process of homogenisation.

These findings also agree with the results of Morel et al. (1971) who studied duck reticulocyte 15s mRNP complexes after dissociation of the polysomes with EDTA in low salt concentrations. Although the

composition of the mRNA-protein particle is complex, two main proteins of molecular weight 73,000 and 49,000 were obtained. Diffuse minor bands occur in the 52,000 to 64,000 molecular weight range together with three other sharp bands in the 86,000 to 120,000 range (Morel et al. 1973). The two major proteins are phosphorylated, as is the diffuse minor component of molecular weight 64,000. The authors have also produced several lines of evidence that suggest the mRNA-protein complexes are not of an artificial nature. No aggregation occurs between the 9s globin mRNA and the 15s mRNA-protein complex when they are co-centrifuged. If the particle is dissociated with sodium dodecylsulphate the absorbance at 260nm shifts to 9s, coincident with 9s mRNA prepared from polysomal RNA. When analysed on iso-kinetic sucrose gradients containing various concentrations of LiCl, they are stable/ yet the ionic strengths fully dissociated artificial RNA-protein complexes. Gel electro-phoretic analysis of the mRNP samples on exponential polyacrylamide gradients indicated that they were discrete particles, since the specific proteins, including the minor components, remained attached during electrophoresis and the separation pattern was not altered when the mRNP was purified by further zonal centrifugation. By using the new technique of dark field electron microscopy, the same group could clearly differentiate between mRNA-protein complexes and mRNA (Dubochet et al. 1973). When they compared mRNA and mRNP from rabbit reticulocytes and duck erythroblasts, all appeared as linear structures 170-220nm. long. However the mRNP particles could be visualised with uranyl acetate at one thousandth of the concentration used to detect mRNA. The authors visualised 4-7 distinct areas on the mRNP stained with I mM uranyl acetate which they believe may represent the proteins bound at discrete sites along the length of

the globin mRNA, possibly in regions of high secondary structures. When mRNA was stained with I mM uranyl acetate, similar blobs (5-8 in number) were evident but disappeared almost completely when the mRNA was denatured, whilst the mRNA showed a 25% elongation. These blobs detected on mRNA may represent staining artefacts or sites of secondary structure. The defined structure of the complex as viewed by dark-field microscopy, and its difference in staining properties from mRNA, is a strong argument for the reality of specific, discrete mRNA-protein complexes.

An independent line of investigation, again employing ionic conditions that minimise non-specific binding of cytoplasmic proteins to mRNA, suggested that four major polypeptides were attached to the mRNA of KB cells (Lindberg and Sundquist, 1974). The mRNA-protein complexes from EDTA dissociated polysomes were isolated by affinity chromatography on oligo (dT)-cellulose, in 0.2M NaCl. The approximate molecular weights of the proteins, as determined by SDS-polyacrylamide electrophoresis, were 56,000, 68,000, 78,000 and 130,000. When the KB cells were infected with adenovirus, and the mRNP harvested late in the infectious cycle, then an extra protein possibly virus-specific, (molecular weight 110,000) could be detected in addition to a set of proteins with identical migration values to those of the uninfected cells. An attempt to compare globin and lens mRNPs isolated by this method with similar mRNPs obtained by EDTA induced dissociation of polysomes is also reported here, in studies aimed at characterising further the nature of messenger ribonucleoprotein particles.

To summarise, when this investigation began there was a distinct possibility that ribonucleoprotein complexes arose through artificial RNA-protein associations. Subsequent biochemical refinements to

mRNP extraction techniques, particularly the use of high ionic conditions, have reduced considerably the amount of non-specific adsorption of protein to RNA, although fortuitous binding is a common finding. The most recent evidence compiled with a variety of techniques from a wide range of animal tissues strongly suggests that mRNP complexes are not merely artefacts of isolation procedure. This evidence has been amassed in the introduction to this investigation to underline the biological reality and significance of mRNP complexes.

Chapter 2

MATERIALS AND METHODS

Chemicals

Unless otherwise specified, all chemicals were of the highest purity available from British Drug Houses, Ltd., Poole, Dorset, U.K.

Preparation of Chick Lens Extracts

The heads from freshly killed adult chickens, kindly supplied by D. B. Marshall, Ltd., Newbridge, Scotland, U.K., were transported from the slaughter house in ice buckets. Lenses were extracted from the eye and dissected free of contaminating tissues with watch-maker's forceps. The cleaned lenses were then homogenised in a Potter homogeniser. The homogenates were prepared in 10 mM-phosphate buffer, pH 7.2 containing 10 mM 2-mercaptoethanol, and then centrifuged at 4°C for 15 minutes at 10,000 g. in the MSE 18 centrifuge fitted with the 8 x 50 rotor head. This low speed spin removed any iris contaminants, cell nuclei and cellular debris. The supernatant was decanted off, its approximate concentration was estimated by refractometry (Clayton et al. 1968) and the solution then stored at -15°C until required.

Day old chicks were kindly supplied by Sterling Poultry Products Ltd., Ratho, Scotland, U.K., and extracts were prepared in an identical manner to the adult material. Lenses required for polysome preparations were immediately stored in liquid nitrogen after extraction, until required.

Polyacrylamide Gel Electrophoresis

a) Non-dissociating Conditions

The technique used was described by Truman (1968), (derived from the method of Ornstein (1964) but without sample or spacer gel). A

discontinuous buffer system was employed, the gel being made up in 0.38 M Tris buffer with the pH adjusted to pH 8.9 by the addition of HCl. Both electrode buffers were 0.038 M glycine solution adjusted to pH 8.3 by the addition of solid Tris. The gel solution was prepared by dissolving 6.82 gm. of acrylamide and 0.184 gm. of N:N'-methylene-bis-acrylamide in 99ml. of the pH 8.9 buffer. 30 ml. of TEMED (N:N:N':N'-Tetramethyl-1:2-diamino-ethane) and 78 ml. of 2-mercaptoethanol were added and the solution filtered through Watman's No. 1 filter paper. Polymerisation was normally effected by the addition of 0.3 ml. of a freshly prepared 7%(w/v) solution of ammonium persulphate to 29.7 ml. of the gel solution. This volume was sufficient to make up to 20 gels. Both Truman (1968) and Day (1971) found more consistent results with this method, as compared to polymerisation by the addition of riboflavin followed by irradiation with ultra-violet light.

The gels were polymerised in glass tubes 75 mm. x 5 mm. diameter. The apparatus used was similar to that of Davis (1964). 16 gels could be electrophoresed at one time, all equidistant from the platinum electrodes. The volume of each electrolyte buffer was approximately 900 mls. per tank, with an air space of about 2 cm. between the upper and lower electrode buffers. All runs were performed at room temperature without any cooling device.

Usually the sample was applied by microsyringe to the top of the gel rods, in volumes of 10-15 ul. Where necessary a few grains of sucrose were added to give satisfactory layering. The run was started at 50 V. for 15 minutes, while the sample entered the gel, and then the voltage was increased to 300v. until the buffer front was near the end of the gel. This took something like 80-90 minutes at the higher

voltage. The current was never allowed to exceed 2 mA per tube.

The gels were stained for protein by various methods as detailed in the text. When stained with Amido-black (napthalene black, G.T. Gurr Ltd.) the gels were soaked in a 1% solution made up in 7% acetic acid for 60 minutes, then left for 2-3 hours in 7% acetic acid before destaining by electrophoresis perpendicular to the length of the gel. Gels were stored in 7% acetic acid.

b) Dissociating Conditions

When electrophoresis was carried out in the presence of urea, the sample was dissociated in 8m-urea, either by dialysis overnight against 8M urea (particularly polyacrylamide slices) or dissociated immediately with 11-M urea, adjusted to give a final concentration of 8M urea. Urea was added to the acrylamide gel solution to give a final concentration of 6M urea. Similarly the upper (cathodal) vessel of the electrophoresis tank contained buffer with urea to a concentration of 6M.

Identical running conditions to the procedure were used in the absence of urea but the final running time was approximately two hours.

Precipitation of Proteins with Ammonium Sulphate

The procedure of Clayton (1969) was followed exactly. When polyacrylamide slices were required for re-electrophoresis or other purposes, protein bands were precipitated *in situ* with ammonium sulphate. Polyacrylamide gels were placed in an 80% saturated solution of ammonium sulphate containing 10mM 2-mercaptoethanol, at 4°C for some 30-60 minutes until precipitate bands could be seen. Oblique illumination against a dark background was used to detect minor components. The gels were placed on a glass plate supported a few inches above a small black disc in the centre of an X-ray viewing box, and the protein bands cut out individually, as thin slices, with a razor blade.

Analysis of the Proteins Associated with the RNA separated from EDTA-treated Polyribosomes by Sucrose Gradient Centrifugation

Chick lens polysomes were dissociated in a low salt buffer containing 33mM. EDTA and then subjected to sucrose gradient centrifugation. The protein moieties of the ribosomal sub-units and of the small RNP complex released from the polysomes by this chelation procedure were then analysed on acrylamide-SDS gels and compared to the proteins released from polysomes by high salt concentrations or sodium deoxycholate treatment.

Preparation of Polysomes prior to Sucrose Gradient Centrifugation

A batch of 140 lenses of one day old chicks was homogenised in 0.25 M sucrose made up in medium A 50 mM NaCl-80 mM KCl 0.5 mM Mg_2SO_4 $6H_2O$-50 mM Tris.HCL (pH 7.2). Cellular debris was removed by centrifugation at 15,000 rev/min. for 20 minutes. The post-mitochondrial supernatant was then layered over 2ml. 0.5 M sucrose in the same low salt buffer and spun at 4°C. in the 3 x 20 rotor head of the M.S.E. Superspeed 50 centrifuge at 25,000 rev/min. (= 70,000 xg) for 16 hours.

Sucrose Gradient Centrifugation

The ribosome pellet (6 A_{260} units, i.e. 300 µg. RNA) was then taken up in 50mM Tris.HCl(pH 7.4) in a total volume of 1.62 ml. Aliquots containing approximately 3 A_{260} units were then made 33mM with respect to EDTA, and applied in a final volume of 1.08 ml. to 15-30% sucrose gradients made up in 50 mM Tris.HCl (pH 7.4). The gradients were then centrifuged in the MSE 3 x 20 rotor at 4°C for 6.5 hours at 30,000 rev/min. (=100,000 xg). 15 drop fractions were collected and their adsorbance at 260 nm determined in a Beckman DB spectrophotometer. Selected fractions were then pooled and mixed

with 2 volumes of ethanol. After 24 hours at -20°C. precipitates were collected by a low speed centrifugation of 10,000 rev/min. for 20 minutes at 0°C.

Isolation of Polysomes from Chick Lens prior to High Salt or Sodium Deoxycholate Treatment

Lenses of one day old chicks, in batches of 400, isolated as described above, were allowed to lyse for 10 minutes in 30 mls of ice-cold extraction buffer containing 0.15 M KCL, 5mM $MgCl_2$ and 50mM triethanolamine (pH 7.5 at 20°C.) and 0.1% diethyl pyrocarbonate. The lenses were then quickly homogenised for 3-4 minutes in a tight fitting glass container. Cellular debris was removed by centrifugation at 15,000 rev/min. for 15 minutes. The post-mitochondrial supernatant was then carefully layered over a cushion of 2 mls of 1.5 M sucrose made up in the extraction buffer. The polysomes were then sedimented through the sucrose at 50,000 rev/min. (=155,000×g) for 3 hours at 4°C. in the 10 x 10 rotor head of the MSE Superspeed 50. The centrifuge tubes were then inverted and drained for two minutes in air. The polysome pellet was then gently dislodged from the side of the tube with a small piece of glass rod and resuspended in an appropriate buffer.

Removal of Proteins from Polysomes by Treatment with Sodium Deoxycholate

This was essentially carried out according to the method of Olsnes (1971, c.). A suspension of chick lens polysomes (approx. $15A_{260}$ units) was resuspended in extraction buffer to which sodium deoxycholate had been added to a final concentration of 1%. After standing for 30 minutes at 0°C., 8 mls of this preparation was then carefully layered onto a 2 ml. cushion of 1.5 M sucrose in extraction buffer and centrifuged in the 10 x 10 rotor head of the MSE Superspeed 50 at 4°C. for 3 hours at 50,000 rev/min.

The upper 4mls. of the gradient, corresponding to the protein solubilised by the sodium deoxycholate, was then carefully pipetted off and added to 16mls. of ethanol. With ethanol at this final concentration of 80% the detergent remains in solution. After 24 hours at -20°C. a precipitate was collected by centrifugation at 15,000 rev/min. for 20 minutes at 0°C. The precipitate was then prepared for electrophoresis in polyacrylamide-S.D.S. gels. This preparation is referred to in the text as the 'deoxycholate wash fraction'.

Removal of Proteins from Polysomes by High Salt Concentrations and Preparation of Salt-washed Ribosomes

This was essentially carried out according to the method of Blobel (1972). A ribosome pellet, prepared from chick lenses was resuspended in a high salt buffer containing 0.5 M KCL, 5mM $MgCl_2$ and 50 mM triethanolamine (pH 7.5 at 20°C.) and 0.1% diethyl pyrocarbonate. Eight mls. of this preparation was then carefully layered over a cushion of 2mls. of 1.5 M sucrose made up in the high salt buffer. The polysomes were then sedimented through the sucrose at 50,000 rev/min. for 3 hours at 4°C. in the 10 x 10 rotor head of the MSE Superspeed 50 Centrifuge. The upper 4 mls. of the gradient were carefully pipetted off, and ethanol added to a final concentration of 80%. After 24 hours at -20°C. a precipitate was collected by centrifugation at 15,000 rev/min. for 20 minutes at 0°C. The precipitate was then prepared for electrophoresis in polyacrylamide-SDS gels. This preparation is referred to in the text as the 'high salt wash fraction'. The pellet of salt washed ribosomes was then used for the isolation of mRNP or prepared for electrophoresis in polyacrylamide-SDS gels.

Zonal Ultracentrifugation

Serious limitations to density-gradient centrifugations are imposed by the capacity and geometry of swing-out rotor tubes, expecially in high speed rotors. Zonal centrifugation overcomes a number of the disadvantages of the swing-out bucket technique. The capacity is considerably larger, so larger amounts of material can be analysed. Because sedimentation occurs in sector-shaped compartments there are no wall effects to effect resolution in the density gradient, whilst the large volume of gradient permits the design of gradients for optional separation of particular components.

Convex sucrose gradients were automatically prepared by the 11300 Ultro-grad gradient mixer (L.K.B. Instruments Ltd., South Croydon, U.K.) generally over the range 10-40% sucrose. Centrifugation was carried out on the B-XIV zonal rotor head (M.S.E. Ltd., Crawley, U.K.) using the M.S.E. Superspeed 65 centrifuge, (M.S.E. Ltd., Crawley, U.K.), at the specified speeds within the range 35,000 to 42,000 rev/min. (60,000 to 85,000 Av. g.). All sucrose solutions were made up in 0.02 Tris, pH 7.5, treated overnight at 4°C. with 0.1% diethyl pyrocarbonate (B.D.H.) to remove RNase activity (Williamson, 1970). Zonal ultracentrifugation was performed according to the manufacturer's instructions (M.S.E. Technical Publication No. 49, M.S.E. Ltd., Crawley, U.K.) and the guide for novices to zonal work (Williamson, 1971).

At the end of the run, generally 14 to 17 hours later, as specified in the text, the gradient was removed from the rotor according to the manufacturer's instructions, by pumping in a solution of 40% sucrose whilst the rotor was slowed to 2,000 rev/min. (1,000g.). Adsorbance at 260 nm was monitored by a Unicam SP-800 spectrophotometer, (Pye, Unicam Ltd., Cambridge, U.K.) using a Perkin-Elmer variable path length

UV flow cell.

Amplification of results was performed with an Servoscribe chart recorder. 130 drop fractions (approx. 11 mls.) were collected by use of the Ultrorac fraction-collector (L.K.B. Instruments, South Croydon, U.K.), the required fractions pooled, made 0.4 M with respect to sodium chloride, then precipitated overnight at -20°C. with two volumes of absolute alcohol.

The Analysis of Protein Samples by Electrophoresis in Sodium Dodecyl Sulphate-Urea-Acrylamide Gels

Various protein samples, in particular the protein moiety of RNP particles, were analysed according to the gel technique described by MacGillivray et al. (1972). This utilises a modification designed by Laemmli (1970) whereby sodium dodecyl sulphate is employed in the double-gel discontinuous electrophoresis procedure of Davis (1964). Electrophoresis was carried out in 4M urea, 0.1% SDS, 15% polyacrylamide. The presence of urea ensured that all proteins were fully dissociated. The gels were prepared from the following stock solutions:

Lower small pore running gel

Solution A: Tris 36.6g.; N,N,N¦N¦ - tetramethylenediamine (TEMED) 0.23mls IM HCl 48ml; made up to 100mls with water (final pH 8.9)

Solution B: 40% acrylamide 0.6% bisacrylamide.

Solution C: 0.14% Ammonium persulphate in 8.M urea, 0.2% SDS.

Upper large pore stacking gel

Solution D: Tris, 5.98 gm. TEMED, 0.46 ml., titrated to pH 6.7 with 1M HCl and made up to 100 ml. with water.

Solution E: 10% Acrylamide, 2.5% bisacrylamide.

Solution F: riboflavin, 4mg. in 100ml. of water.

Solution G: 8M urea, 0.2% SDS.

The small pore gels were prepared by mixing 1 vol. of solution A with 3 vols. of solution B and 4 vols. of solution C. (A final volume of 32 ml. provided ample material for a tank load of 16 gels.) Aliquots of 1.5 ml. were then carefully pipetted into 11 cm or 12 cm glass tubes and overlaid with water or iso-butanol, after air bubbles had been removed by gentle tapping. After a polymerisation time of approximately 1 hour, the water or iso-butanol layer was removed by a fine 1ml. pipette and replaced by 0.2ml. of the large pore stacking gel solution. This consisted of 1 vol. of solution D with 3 vol. of solution E, 1 vol. of solution F and 4 vol. of solution G. The gels were then photopolymerised for 1 hour under a fluorescent tube. The total length of the polymerised gel was usually 9 cm. In some cases slightly longer gels of 9.5 cm. were employed.

Preparation of Samples for SDS Electrophoresis

The proteins were dissolved in 8 M urea, 1% SDS, 1% 2-mercaptoethanol, 10mM Tris (pH 7.5) and incubated at 37°C for 3 hours. They were then dialysed overnight against 8M urea, 0.1% SDS, 1% 2-mercaptoethanol in 10mM Tris (pH 7.5). Samples of each solution in a maximum volume of 200 µl. were then mixed with 5µl. of 2-mercaptoethanol and 5µl. 1% bromophenol blue marker dye and loaded onto the gels after the water overlay had been removed from the gels.

Electrophoresis was carried out at room temperature at 2 mA/gel in a buffer system of 3gm. Tris, 14.4 gm. glycine and 1 gm. of SDS per litre. After the marker dye had reached the bottom of the small pore gels (approx. 2.5 hours), final voltage 250V., the gels were removed from the electrophoresis apparatus and the glass tubes cracked in a vice. The length of the gel and the distance moved by the marker dye were measured. Each gel was then fixed overnight in methanol: acetic acid: water (5:1:4:v/v).

Staining of gels

Initially, gels were stained for 2 hours with 1% (w/v.) Napthalene Black (Amido Black) in 7% (v/v) acetic acid and destained electrophoretically (400 ~~eross~~/volts) in 7% acetic acid.

A second method of staining with 0.2% (w/v) Coomassie Blue, identical to the method described for the iso-electrofocussing gels, (Chapter 3) was adopted for later experiments, because of the increased sensitivity of staining available with this dye. Thus gels were stained in 0.2 (w/v.) Coomassie Brilliant Blue in 10% (v/v.) acetic acid and 25% (v/v.) methanol for 30 minutes at 65°C.

Destaining was performed exactly as described for iso-electric focusing in polyacrylamide gels, (see Chapter 3).

Estimation of Molecular Weights

A calibration curve was constructed by running proteins of known molecular weight on SDS-urea acrylamide gels. The following marker proteins were employed in molecular weight determinations: cytochrome C (horse heart), ovalbumin (chicken egg), and β-lactoglobulin (bovime) purchased from Koch-Light Ltd., Colnbrook, Bucks., England.

Bovine serum albumin, aldolase and galactosidase (E. coli) were provided in the Boehringer Combithek Calibration set, Boehringer Mannheim, Gumb H, West Germany.

TABLE 5

VALUES ACCEPTED FOR MOLECULAR WEIGHTS OF THE POLYPEPTIDE CHAIN OF THE STANDARD PROTEINS

PROTEIN	MW. POLYPEPTIDE CHAIN	REFERENCE
beta-galactosidase	130,000	Weber and Osborn (1969)
alpha-phosphorylase	94,000	" " " "
bovine serum albumin	68,000	" " " "

catalase	60,000	Weber and Osborn (1969)
ovalbumin	43,000	" " " "
aldolase	40,000	" " " "
chymotrypsinogen	25,700	" " " "
beta-lactoglobulin	17,500	Klotz (1967)
cytochrome C	12,400	Andrews (1965)

Calculation of Electrophoretic Mobilities

The gels swell considerably in the course of staining and de-staining. Weber and Osborn (1969) found that gels showed more swelling as the amount of cross linker was lowered. In these urea gels the ratio of acrylamide to methylene bis acrylamide was 67:1, whilst Weber and Osborn (1969) generally employed twice this amount of cross linker. Consequently it was important in estimating electrophoretic mobilities to allow for this swelling effect. Mobilities were calculated according to the formula suggested by Weber and Osborn (1969).

$$\text{Mobility} = \frac{\text{Distance of Protein Migration}}{\text{Length after destaining}} \times \frac{\text{Length before starting}}{\text{Distance of Dye Migration}}$$

The distance of protein migration was always measured from the leading edge of the protein band. The mobilities were then plotted against the known molecular weights expressed on a semi-logarithmic scale.

The general shape of the calibration curve obtained in this manner was hyperbolic, in good agreement with the results of Weber and Osborn (1969) where they used a similar low amount of cross linker. In general the separation on the molecular weight range 30,000-60,000 was very good. Unfortunately, fewer markers are available in the higher molecular weight range where the curve is steeply hyperbolic. Consequently greater reservation must be made about any estimations in the range 55,000-130,000. Reproducibility however was good when care was taken not to overload the gels. The following amounts of marker proteins

were routinely employed:

beta-galactosidase	100µg	in	100µl
alpha-phosphorylase	10µg	in	20µl
bovine serum albumin	20µg	in	20µl
catalase	10µg	in	20µl
ovalbumin	10µg	in	2µl
aldolase	30µg	in	30µl
chymotrypsinogen	20µg	in	20µl
beta-lactoglobulin	10µg	in	2µl
cytochrome C	10µg	in	2µl

Preparation of High Salt Washed Ribosomes from Mouse Reticulocytes

Mouse blood, of high reticulocyte content, was taken from storage at -70°C. and allowed to lyse for 5 minutes in an equal volume of ice-cold I mM $MgCl_2$ solution containing 0.1% diethyl pyrocarbonate, before being homogenised by hand in a tight fitting glass container. Cellular debris was removed by two successive low speed centrifugations of 10,000 rev/min. (approx. 10,000g.) at 4°C for ten minutes. The post mitochondrial supernatant was then carefully layered by pipette onto a 2 ml. cushion of I M sucrose made up in 100 mM Tris-HCL, pH 7.4, 1 mM $MgCl_2$, 0.1% diethyl pyrocarbonate. Polysomes were collected by centrifugation at 55,000 rev/min. (235,000 x g.) for 2 hours in the 8 x 25 Ti rotor head of the M.S.E. 65 Superspeed centrifuge at 4°C.

The polysome pellets were then quickly homogenised, in the cold (2-4°C.) in 10ml. of high salt buffer (Blobel, 1972). This consisted of a solution of 500 mM KCL, 50mM Tris-HCL, pH 7.4, 5 mM $MgCl_2$, 2 mM DTT, 0.1% diethyl pyrocarbonate. The ribosomal solution was then layered over a 2 ml. cushion of 30% sucrose made up in the same buffer. A second pellet of high salt washed polysomes was then collected by centrifugation at 55,000 rev/min. for two hours in the 8 x 25 Ti rotor

head of the M.S.E. 65 Superspeed centrifuge at 4 °C. The polysome pellets were then quickly washed in high salt buffer prior to puromycin dissociation.

Dissociation of Mouse Reticulocyte and Chick Lens Ribosomes by Salt Puromycin Treatment

This was again carried out according to the method of Blobel (1972). To each pellet of salt washed ribosomes (usually about 85 A_{260} units) 3 ml. of a solution of 500 mM KCL, 50 mM Tris-HCL, pH 70, 2 mM $MgCl_2$, I mM DTT, - I mM puromycin was added. The samples were incubated at 37 °C. with occasional gentle swirling, then quickly homogenised in a small tight fitting homogeniser, then allowed to stand for a further 3 minutes at 37°C. A slightly long period of homogenisation was utilised for chick lens polysomes, to transform the pellet into a clear solution of dissociated ribosomes.

Preparation of Columns for Affinity Chromatography

Small chromatography columns were made of 5 ml. syringe tubes, 115 mm. in length, 11 mm. internal diameter. They were charged with oligo(dT)-cellulose, (Searle, High Wycombe, Bucks., U.K.) washed in equilibrium buffer, 0.2 M NaCl, 50 mM Tris-HCL, pH 7.8, 10 mM EDTA, 0.2% NP40. The slurry was applied to the syringe tube and allowed to pack onto a small glass fibre support in each column, to a final height of about 25 mms. The column was then washed repeatedly with 0.1 N NaOH and then with equilibration buffer, again repeatedly, before being stored at -20°C. prior to use.

Affinity Chromatography on Oligo(dT)-cellulose

Polysomes, whether dissociated with EDTA at a final concentration of 33 mM EDTA, pH 7.4 or high salt washed polysomes dissociated by puromycin, were mixed with 4 volumes of equilibration buffer before

being applied to the column. The unadsorbed fraction was then eluted with 15 ml. of equilibration buffer. The retained fraction was then eluted by 6 ml. of 25% formamide buffer: 0.2 M NaCl, 50 mM Tris-HCL, pH 7.8, 10 mM EDTA, 25% formamide, followed by 6 ml. of buffer of the same composition except for a final concentration of 50% formamide. Occasionally, a similar amount of 90% formamide buffer was also applied. Any remaining adsorbed material was eluted with 5 ml. of 0.1 N NaOH.

All eluates were then made 0.4 M with respect to NaCl, before being precipitated overnight at -20°C. with 2 volumes of ethanol.

Obtaining and Labelling Embryonic Lenses

Embryos

Fertilised eggs were purchased from D. B. Marshall Ltd., Newbridge, Midlothian, Scotland, U.K. The eggs were incubated at 37°C. and staged by the morphological criteria described in Lillie's Development of the Chick (1952).

Radio-active Chemicals

These were obtained from the Radiochemical Centre, Amersham, England, U.K. The ^{3}H-amino-acid mixture was supplied at a specific activity of 1 mCi/ml. and the ^{14}C amino acid mixture at 50μCi/ml.

Dissection and Culture of Lenses

Dissection of embryos was carried out under a Horizontal Laminar Flow Bench (South London Electrical Equipment Company, Surrey, U.K.) to minimise bacterial contamination, and normal laboratory sterile procedures were observed.

The embryos were staged under a binocular microscope and the intact lenses dissected out by fine watchmakers forceps and cataract needles. In embryos earlier than 96 hours the preparation probably also contained optic cup tissue. Small pieces of iris clinging to the lens could be easily removed in all but the oldest embryonic stages.

Pre-radioactive Incubation Period: Lenses were cleaned of adhering

debris in pre-warmed medium 199 (Wellcome Research Laboratories, Beckenham, England, U.K.) containing 200 units/ml. of penicillin (Flow Laboratories Ltd., Irvine, Scotland, U.K.) before being transferred to pre-warmed supplemented medium. Identical numbers of lenses were transferred into plastic tissue culture dishes (60 x 15 mm, Falcon Plastics, Oxnard, Ca., U.S.A.) containing either 5 ml. Medium 199 supplemented with 10% (v/v) foetal calf serum (Gibco-Biocult, Glasgow, Scotland, U.K.) and 200 units/ml. penicillin or to the same medium to which actinomycin D (Calbiochem Ltd., London, U.K.) had been added to a final concentration of 15 µg./ml.

All lenses from embryos of 4 days and older were maintained in these conditions for 4 hours at 37°C. in a humidified atmosphere of 95% air:5% CO_2. Younger lens material was pre-incubated for the times specified in the text.

Labelling Period: lenses pre-incubated in medium containing actinomycin D were labelled for 4 hours with ^3H amino-acids (150µCi/ml. in 3 ml. of Medium 199 plus 10% foetal calf serum plus penicillin (200 units/ml.) and actinomycin D (10 µg./ml.). The radioactive material was pre-warmed in plastic tissue culture dishes (35 x 10 mm., Falcon Plastics, Oxnard, Ca., U.S.A.). Similarly the corresponding untreated batch of lenses was labelled for 4 hours with ^{14}C amino-acids (15µCi/ml. in 3 mls. of identical medium but lacking actinomycin D). All lenses were maintained at 37°C. in a humidified atmosphere of 95% air:5% CO_2. At the end of the pulse period the lenses were briefly washed in cold Medium 199 before incorporation was stopped by freezing the lenses with liquid nitrogen. In order to minimise experimental differences arising through homogenisation procedures, both control and actinomycin D treated lenses were collected together and stored, until required, in liquid nitrogen.

Homogenisation Procedure

Embryonic lens material was homogenised in extremely close fitting micro-homogenisers, in a minimum volume of 8 M-urea, 100 mM 2-mercaptoethanol, 10mM-Tris HCL (pH 7.5). The homogenate was then frozen and thawed out from -20°C, repeatedly over a period of 3 hours to ensure maximal cellular breakdown. Cellular debris was then removed by centrifugation at 10,000 rev/min (=11,500 x g) for 10 minutes at room temperature, in the M.S.E. 18 centrifuge. The supernatant was removed and stored at -20°C prior to analysis by gel electrofocusing.

Processing of Polyacrylamide Slices for Scintillation Counting

Polyacrylamide slices (approx. 1 mm. thick) were solubilised overnight in scintillation vials in 0.5 ml. of a 10% (v/v) solution of 100 vols. hydrogen peroxide. To each vial was added 10 ml. of Triton-toluene scintillation mix, to give a clear counting solution. The scintillation mix was made up, per litre, of 700 ml. of toluene, 300 ml. of Triton X 100, 5 gm. of PPO (2,5-Diphenyloxazole) and 0.3 gm. of Dimethyl POPOP (1,4 bis[2(4-methyl-5-Phenyloxazolyl)]-Benzene). All these scintillation chemicals were obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks., England, U.K.

To minimise chemiluminescence and light excitation effects, all vials were stored in the dark at 4°C for a minimum of 16 hours prior to being counted. Slices containing the origin of polyacrylamide gels were not processed.

Double Chanel Counting

Counting of radioactivity was performed in the ABAC SL40 scintillation spectrometer (Intertechnique, Paris, France.). The external standard mode was employed for dual counting of ^{3}H and ^{14}C labels, and the manufacturer's instructions used to derive and store

quench curves, in order to obtain absolute activities for each isotope in disintegrations per minute (D.P.M.). A computer feature was selected so that results were printed out in the form of two separate histograms, in D.P.M., for each of the isotopes, and a third table of isotope ratio. By selecting the correct histogram feature, the radioactivity in any one slice could be expressed as a percentage of the total radioactivity entering the gel.

Immuno-electrophoresis

Immunoelectrophoresis involves two distinct and separate procedures. In the first process the sample is subjected to electrophoresis in a suitable conductive solvent (first dimension). In the second process of immunodiffusion antibodies are allowed to diffuse perpendicular to the antigens separated in the running gel. Precipitates are deposited between the antibody trough and the regions of highest concentration of the electrophoretically separated components. Gelatin agar, agarose and polyacrylamide gels are commonly used to stabilise the conductive solvent in the electrophoretic step and are suitable media for immunodiffusion. These media vary considerably in their endosmotic properties, i.e. the degree of solvent flow through the supporting substance.

Agar and agarose gels are prepared in an identical manner in this laboratory. Agarose is a neutral polysaccharide however, hence the gel is theoretically free from ionised groups and produces a less pronounced endosmosis than agar (Quast, 1971). This fact is of crucial importance where prolonged electrophoretic times are employed but for the micro-electrophoresis technique, described below, agar is totally satisfactory and has the advantage of being relatively inexpensive.

Micro-immunoelectrophoresis in 1.5% agar or agarose.

Immuno-electrophoresis was carried out according to the micro-method of Scheidegger (1955) using the High Resolution Buffer (pH 8.9) of Aronsson and Gronwall (1957). This consists of 60.5 gms./litre of Tris, 6 gm/litre E.D.T.A., 4.6 gm/litre Boric acid, final pH 8.9, diluted before use 1:2 with distilled water. 1.5 gms of agar or agarose were then mixed with 99 mls. of the diluted high resolution buffer in a conical flask. The polysaccharide was then melted by heating the stoppered flask in a domestic pressure cooker for 10 minutes at full steam pressure.

The solution must not be maintained for too long at this temperature, as prolonged heating may induce the partial destruction of gel structures and brownish decomposition products appear.

The melted agar was then transferred from the flask by means of a pre-warmed 10 ml. pipette onto clean microscope slides, supported on a horizontal levelling board (Shandon Scientific Company Limited, London). Each slide was covered with 2 ml. of fluid agar or agarose, care being taken to avoid air bubble formation in the gel. After gel formation, the slides were stored for 1-2 hours at room temperature in a humidity cabinet before use.

The cutting of patterns for immunoelectrophoresis was made by free hand or by means of a commercially available template (Shandon Scientific Company Limited, London). Whenever gels were cut freehand (usually when larger glass plates were employed) the plate was placed over white paper on which outlines representing wells and troughs had been drawn. The sample wells were made with an extended glass pipette and the gel plug removed by continuous suction through the rubber bulb. The antibody troughs

were cut out by means of a scalpel and ruler, tracing out the underlying pattern. The 1mm wide trough was normally cut 5 mm. from the antigen wells.

In routine experiments 2 µl of the protein sample (15 mgs/ml. concentration) was applied to the antigen well by means of a Hamilton syringe. Increased protein concentrations were required on occasions where subsequent separation techniques were employed.

The electrophoretic stage was performed in a workshop built apparatus containing undiluted High Resolution Buffer. Filter paper (3 mm. Whatmans Filter Paper) was soaked in buffer and laid on both ends of the slide (or plate) to connect the buffer solution in the anode (left) and cathode (right) compartments with the gel surface. The slides thus lay at right angles to the electrodes.

Electrophoresis was carried out for 2 hours at 6.5 V/cm or occasionally for 1 hour at 13 V/cm. The trough for anti-serum was then cut out after the electrophoretic separation and approximately 100 µl of antiserum introduced into the trough. The immuno-diffusion step was allowed to occur over a period of about 16-18 hours, in a humidity chamber at room temperature.

Subsequently the gel was washed free from non-reacted antigen and antibody by several changes of 0.9% /W/V) physiological saline over a period of 24 hours. The salt was then washed out in distilled water for about 24 hours.

Gels were then dried down by applying filter paper (Whatman No.1) to the surface of the gel and allowing them to stand in open air until the following day.

The slides (or plates) were then stained for 30 minutes in a

solution of 0.1% Amido Black (Napthalene Black 106) in ethanol-water-glacial acetic acid (7:2:1, V/V) and destained with a mixture of ethanol-glycerol-water-glacial acetic acid (7:1:1:1, V/V) for about one hour with 2 wash changes.

Note.

All of these particular techniques are routinely employed in this laboratory and the essential details can be found in Campbell, Clayton and Truman (1968). General information and further details of immuno electrophoretic techniques can be found in the excellent laboratory handbook by Clausen (1969).

Macro-immunoelectrophoresis

The development of a practical method for enhancing the separation of lens crystallins into major immunological classes by extended agarose electrophoresis is described in the results section (Chapter 6). However the details of preparation of the gel (other than the buffers employed), cutting of wells and troughs, drying and staining of the gel were identical to those described for micro-immunoelectrophoresis.

Treatment of agarose or agar strips prior to gel electrofocusing.

Using the line of sample wells as a reference point, parallel slices of 0.5 cm or 1 cm were cut out at right angles to the electrophoretic track with the aid of a ruler and a scalpel. Antibody troughs were then cut alongside the remaining control samples on the plate and immuno-diffusion allowed to take place, as described.

The strips of gel were then packed into dialysis tubing (Visking 2 cm) and dialysed overnight against 8M urea containing

100 mM 2-mercaptoethanol. The volume of the dialysate was subsequently reduced to one third of its original volume by drying down in a stream of warm air. The gelatinous, concentrated mass could then be easily transferred and packed down on the iso-electric focusing gel by means of a wide bore Pasteur pipette and rubber teat. The agar or agarose was kept apposed to the surface of the polyacrylamide gel by a pledget of glass wool (Clayton 1969).

A longer pre-focusing time of 2 hours was necessary when re-running of proteins from agarose (or agar) gel was performed. If the gradient was not fully established by the pre-run, it appeared that the agarose (or agar) could extend the pH gradient upwards, drawing ampholines from the polyacrylamide into the polysaccharide gel, making the determination of iso-electric spectra virtually impossible. Apart from this adjustment in pre-running time of the gel, all other conditions for iso-electric focusing were as described in the results section (Chapter 3). The iso-electric spectrum of each agarose fraction was then recorded, representing the subunits present in that particular agarose region, whilst the immunodiffusion studies on control samples indicated the immunological relationships of the undissociated assemblages in any particular agarose region.

Immuno-electrofocusing.

This technique was performed according to the recommendations of Catsimpoolas (1973). Isoelectric focusing in polyacrylamide gel in non-dissociating conditions was performed according to the method described in Chapter 3. The intact gel column was then embedded in the 1.5% buffered agar used for micro-electrophoresis (see above). Trenches were then cut parallel to the gel, at a

distance of 10 mm., and filled with antiserum. The details of preparation of the agar gel, cutting of troughs, development, drying and staining of the agar gel were exactly as described for micro-immunoelectrophoresis.

Electroimmunoprecipitation.

After the completion of iso-electric focusing, the gels were embedded in a thin layer of agarose gel containing antibodies to total lens proteins of the chick. For separation in the second dimension the technique used was that of Clarke and Freeman (1968), as modified by Truman et al. (1972a). Thus the high-resolution buffer of Aronsson and Gronwall (1957) was used, diluted to half-strength in the electrophoresis tank and to one-sixth strength in the 1% agarose gel. Electrophoresis was carried out for 18 hour at 7 V/cm at 4°C. The antiserum concentration was 5%. Only preliminary results are reported since considerable difficulties were encountered with this technique, because of differences in endosmotic properties of the polyacrylamide gel and agarose. Drying down and staining of the gel were performed as described for micro-immunoelectrophoresis.

Polyacrylamide Gel Electrophoresis of RNA.

This was carried out essentially according to the method of Loening (1967). Specially purified acrylamide for electrophoresis was purchased from B.D.H. and bis-acrylamide was re-crystallised from acetone. 2.6% acrylamide gels (0.12% bis-acrylamide) were prepared normally in 7.5 by 0. 63cm. perspex tubes. Other lengths and bores are specified in the text. The gel buffer consisted of 36mM Tris-HCl (pH 7.8), 30mM NaH_2PO_4, 1mM EDTA. The electrophoresis buffer was of similar composition but with 0.2% SDS. Pre-running of gels at 5mA/tube for 20-40mins., at 20°C, allowed the SDS to enter the gel. Samples were loaded with the current at 1mA/tube. At the

start of the electrophoretic run, 2.5mA/tube were applied for 20mins. while the sample entered the gel. The current was then increased to 5mA/tube, and the run continued for 90mins.

Samples were applied by microsyringe, dissolved in electrophoresis buffer and 5% sucrose. The usual size of the sample was 2-20µl. After completion of the run the gels were washed for 30mins. in distilled water, then scanned at 260n.m. in the Joyce-Loebel Chromoscan.

Chapter 3

DEVELOPMENT OF A GEL ELECTROFOCUSING TECHNIQUE

It has been argued above (see Introduction) that if the differentiation of the lens fibre is to be considered as a system involving the activity of a number of different genes then it is the synthesis of the individual protein subunits that must be followed. Exact enumeration and comparison of such subunits would allow estimates to be made of the number of loci producing separate gene products. In turn the validity of such studies will depend on the resolution and sensitivity of the analytical technique employed. Proteins purified by a variety of procedures and judged to be homogeneous by such criteria as enzyme specificity or molecular weight have almost invariably shown heterogeneity when analysed by the very high resolution method of iso-electric focusing (Susor et al.,1969) which can distinguish between very minor differences in molecular charge within protein populations. In fact the number of components resolved by iso-electric focusing of apparently homogeneous proteins was initially embarrassing and the use of this analytical technique was questioned. Only in very few cases, however, has the technique itself been proven to introduce apparent heterogeneity into proteins, for reasons discussed below. On the contrary, iso-electric focusing provides a powerful method for studying protein heterogeneity. One recent paper on the microheterogeneity and allomorphism of proteins points out that the use of this equilibrium separation method has led to the re-examination of the criteria on which protein homogeneity is based (Williamson (A.R.) et al.,1973). Since iso-electric focusing (or electrofocusing) in a sucrose density gradient has

been applied successfully to the preparative fractionation of chick lens crystallins in this laboratory (Truman et al.,1971), it appeared worthwhile to try and develop this technique for the separation and identification of lens crystallin subunits.

Principle of Method

Iso-electric focusing involved the migration of amphoteric molecules such as proteins to the regions corresponding to their iso-electric points in a pH gradient (Svensson,1961,1962, Vesterberg and Svensson 1966.) The iso-electric point(pI) of a protein is defined as that pH value at which the net charge on the molecule is zero, i.e. its electric mobility is zero. At the iso-electric point of the protein the charges present on the amino groups and the carboxyl groups exactly neutralise each other.

Suitable pH gradients for iso-electric focusing can be generated by applying a voltage to a mixture of aliphatic polyamino-polycarboxylic acids, possessing themselves a range of iso-electric points, in solutions stable to disturbance by convection (Vesterberg and Svensson,1966). These carrier ampholytes have been commercially available for some years under the trade name Ampholine (LKB, South Croydon, Surrey, U.K.) They are a mixture of many isomers and homologues of polyamino-polycarboxylic acids, with an average molecular weight between 300 and 600 (Wrigley,1968, Wadstrom et al., 1974). They possess a number of desirable properties (summarised in Vesterberg,1973b) which make them suitable for creating natural pH gradients. These include high buffering capacity, good solubility in water and good conductivity. In addition they can be obtained in a wide range (pH 2.5-11), possess low light absorption at 280 n m and longer wavelengths and can be generally removed from

proteins by simple procedures such as gel filtration and dialysis. Their hydrophilic properties reduce the possibility of the carrier ampholytes binding to hydrophobic sites of proteins.

Recent reviews have appeared on the basic principles of iso-electric focusing (Haglund,1971; Vesterberg,1973). However it may be instructive to consider the behaviour of proteins when subjected to a stable pH gradient assumed to have been obtained between anode and cathode by means of the Ampholine carrier ampholytes. If such an amphoteric molecule, possessing both acidic and basic groups, is introduced to the acidic end of a stable pH gradient, H^+ ions will be available, the protein will capture protons and become positively charged. Consequently in an electric field, it will migrate towards the negative pole or cathode, thereby encountering less acidic conditions. At that pH which equals its pI the net charge on the molecule will be zero, the molecule will be electrically neutral and it will stop migrating and become focused. Similarly if the protein is subjected first to basic conditions, OH^- groups will be available, the protein will lose protons, from the carboxylic groups, and have a negative net charge. Consequently it will be transported by the current towards the anode, encounter less basic conditions and again focus at that pH value at which its net charge is zero. Hence any protein introduced to a stable gradient will become focused at that point in the gradient where the pH is equal to the pI of the protein.

Iso-Electric focusing in Polyacrylamide Gel.

Wrigley (1968) described a method of iso-electric focusing in Polyacrylamide gels, where the gel stabilises the pH gradient but

does not serve as a molecular sieve. This method of electrofocusing appeared to offer a number of advantages compared to the use of column iso-electric focusing in a sucrose density gradient. It is a much more rapid technique (3-16 hours as compared to 48-96 hours), avoids the use of large amounts of reagents and sample, and above all, allows numerous samples to be analysed together in the same run. Detection, preservation and recording of results are also much simpler since staining, photographic and protein quantitation techniques developed for gel electrophoresis methods are all available.

Two methods of iso-electric focusing in polyacrylamide gel appear to have been developed independently in several laboratories. Many publications describing a gel-rod procedure appeared in 1968 (Wrigley,1968; Riley and Colman, 1968; Fawcett 1968; Dale and Latner, 1968). At the same time the first descriptions appeared of iso-electric focusing of proteins in thin layers of polyacrylamide gel (Leaback and Rutter,1968; Awdeh et al.,1968). Soon after this latter technique was used to analyse chick lens crystallins (Bours and van Doorenmaalen,1970). The thin layer method has a number of advantages including direct and easy comparison of multiple samples, ease of photography and more efficient heat exchange. Furthermore iso-electric points can be estimated from thin layer gels more accurately than gel electrofocusing in cylindrical tubes (Wrigley,1968). On the other hand the gel rod procedure is more suitable for re-focusing experiments, slicing for radio-active counting and can be more readily used for subsequent immunological analysis (see review ~~schemata~~ on immuno-isoelectricfocusing, Catsimpoolas, 1973). Larger amounts of protein can be applied to gel rods, whilst there are difficulties in applying proteins on flat-bed

gels (Vesterberg,1973a). However it was decided to employ the gel rod procedure mainly because conventional apparatus for gel rod electrophoresis was available, that required no further modification for use in electrofocusing.

General features of the developed technique.

In order to study the subunit composition of lens crystallins, 6M urea was incorporated into the ampholine gels. Wrigley (1968) first employed 2M urea when focusing gliadin proteins, subsequently many authors have employed urea in concentrations from 6 to 9 molar, for a wide range of protein samples (Catsimpoolas,1969; Florini et al.,1971; Salaman and Williamson,1971; Merz et al.,1972).

The final modifications of the basic technique described by Wrigley (1968) that were systematically introduced for the gel electrofocusing of crystallin subunits are described in detail below. Since the development of this technique was systematic and gradual, where applicable the rationale for particular experimental procedures has been outlined in the text below. However apart from employing 6 M urea, the two major features of the modified technique are as follows:

1) The use of anti-oxidant chemicals in all dissociation and separation procedures to prevent oxidation of susceptible side-groups within polypeptides, that could otherwise result in the recording of artefacts, particularly through interchain disulphide bonding (Catsimpoolas,1969; Salaman and Williamson,1971).

2) The admixing of carrier ampholines of different pH ranges to obtain a pH gradient that will maximally separate a set of proteins with iso-electric points in the range pH 3-10. This

technique can greatly aid the analysis of protein complexity. Since most proteins have their iso-electric point within this range of pH 3-10, the modifications introduced appear to be generally applicable to the analysis of many developmental systems.

Gel Electrofocusing : Experimental Details

Preparation of acrylamide Gels

A 7.5% (W/V) polyacrylamide gel containing 1% (W/V) "Ampholine" carrier ampholytes (LKB - Produckter AB, South Croydon, Surrey, U.K.) was prepared according to the method of Wrigley (1968), with modified stock solutions so that a final concentration of 6 M urea was obtained in the gel. Further details of the procedure together with various other modifications are given below. Stock solutions for preparing the gel were as follows:

A) Catalyst solution : I ml. N,N,N^1,N^1 - tetramethyl 1-2 diameothane (TEMED), distilled water to 100 ml.

B) Acrylamide solution : 30 gm. acrylamide, 0.8 gm. N-N' ethylene bisacrylamide, distilled water to 100 mls.

C) Chemical polymerisation solution : 80 mg. ammonium persulphate 43.2 gm. urea, distilled water to 80 ml. i.e. I% (W/V) of ammonium persulphate in 9 molar urea.

All of the solutions described were filtered prior to use. If stored in dark containers at 4°C all of the solutions could be used for at least four weeks following preparation, but the ammonium persulphate catalyst in urea was routinely prepared as required and not stored.

Glass tubes of 5 mm internal diameter and either 75 mm or 120 mm long were presoaked in 100 vols. hydrogen peroxide for 12-18 hours

then rinsed in distilled water and finally oven dried at 150°C. The gels were sealed at the bottom and filled to within 1-2 mm of the top with the polymer gel solution. This concentrated gel mixture was prepared by mixing the following proportions of stock solutions :

TEMED catalyst 0.8 ml.

Acrylamide - bis monomer 3.0 ml.

40% (V/V) Carrier ampholine solution 0·3 ml.

This mixture was then degassed with a water pump.

To 4 mls. of this mixture 8 ml were added of the ammonium persulphate-urea solution, which had been similarly degassed. This final volume of the gel solution is sufficient for casting 8 small gels (60 mm lomg) or 5 gels 95 mm in length. After tapping to remove any trapped air bubbles the gels were immediately overlaid with iso-butanol, which since it is virtually immiscible with aqueous solutions, produces a very flat top to the gel in the course of polymerisation. This distinguishes top from bottom of the gel in subsequent manipulations, whilst a flat meniscus can help prevent artefacts in experiments where protein contained in polyacrylamide slices is re-run into ampholine gels. Initial polymerisation occured within 10-15 minuts and the gels were then allowed to polymerise for a further two hours at 4°C before use.

Preparation of Electrode Solutions

The upper electrode (anodal) solution consisted of 0.2% (V/V) sulphuric acid containing 10 mM 2-mercaptoethanol. This reducing agent was added to prevent reassociation of subunits through the interaction of inter-chain disulphide bonds. The addition of a reducing agent

has been recommended to help combat the oxidising effect of the dissolved oxygen or other products with oxidising capability that may be generated at the anode during iso-electric focusing (Vesterberg,1973b). As a further precaution 10 mM 2-mercaptoethanol was also used in the lower (cathodal) electrode solution of 0.4% (V/V) ethanolamine. Both were stored at 4°C for several hours prior to use. Immediately before beginning the iso-electric focusing run, the ethanolamine solution was placed in the lower compartment of an electrophoresis apparatus similar to that described by Davies (1964). The iso-butanol layer was removed by pipette and the gel meniscus flushed with distilled water which was similarly removed. The anodal solution was then added carefully to the upper chamber after the gels had been correctly aligned vertically.

Application of the Sample

The sample (in 8-11 M urea, 10% sucrose, 100 mM 2-mercaptoethanol) was applied to the top of the gel under a protective layer of carrier ampholyte solution containing 5% sucrose, made up as follows:

40% (V/V)	Carrier ampholine solution (3-10 pH range)	.05 ml.
	Sucrose	100 mg.
	2-mercaptoethanol (100 mM)	15.6 µl.
	Water	to 2 ml.

The admixing of the protein sample to the ampholine solution protects the sample from the effects of extreme pH changes and the sucrose layer of intermediate density separates the protein sample from the strong acid electrolyte. Application of the sample at the anode was preferred since there is a possibility that the α-crystallin component of chick lens may precipitate if applied at the cathode

(Bours,1971). Artefacts can occur where proteins are denatured on being subjected to pH values increasingly different from their own respective iso-electric point (Lewin,1970). Whilst such a phenomena may lead to the appearance of additional bands, this source of artefact through denaturation is more likely to be relevant in non-dissociating conditions of iso-electric focusing. However since placing the lens sample (in the absence of 2-mercaptoethanol) directly into the gel before polymerisation was complete yielded a comparable iso-electric spectrum to that obtained where the sample was loaded at the anode, it appears that none of the bands obtained here were due to this type of artefact.

Iso-electric Focusing Procedure

Pre-running of the gel was routinely performed at a constant current of 0.5 mA per tube for 30 minutes at 4°C, in the presence of 50 µl of carrier ampholyte layer per tube. This current sets up the pH gradient and removes catalysts employed in the gel polymerisation (Wrigley,1968). Longer pre-running times of 3-6 hours gave identical results where the sample was applied in solution.

A longer pre-running time of 1-2 hours was found necessary when rerunning of proteins from polyacrylamide slices was performed. When the gradient was not fully established by the prerun it appeared that the polyacrylamide slice could extend the pH gradient upwards, drawing ampholines from the gel into the slice, often with disastrous consequences.

After the pre-focusing period, the sample was then carefully layered on top of the gel, under the carrier ampholyte layer, with a Hamilton microsyringe, generally at a concentration of 250 µg.

per gel. The run was carried out at temperature of 4°C for a further 16-18 hours, during which time the voltage rose from an initial value of 45 volts until the potential difference was finally maintained at about 400-450 volts. The current fell normally in this period to a final value of less than 2 mA for the entire tank.

No heating effects were observed in runs performed at 4°C provided the current had been maintained at a maximum of 0.5 mA per gel. However longer electrofocusing times can lead to instability of the pH gradient, apparently due to the electro-osmotic flow of hydrogen and hydroxyl ions (Finlayson and Chrambach,1971). This "plateau phenomenon" was observed when gels were electrofocused over 22 hours. The focused protein bands appeared to migrate to the cathode and finally leave the gel. Such types of artefact were also observed whenever leakage of the upper electrode to the cathodal compartment occured.

Determination of pH

The pH determinations were made at a temperature of 4°C, the temperature of the electrofocusing run, since the pI values of focused proteins, although independent of the zone-focusing temperature are dependent on the temperature at which the pH measurements are taken (Vesterberg and Svennson, 1966). The difference in iso-electric point of various proteins at 4°C and 25°C ranges from about 0.1 to 0.4 pH unit (Vesterberg and Svennson,1966; Josephson et al.,1971; Bours,1973 c).

The polyacrylamide gels were sectioned, at 4°C, into 0.5 cm slices with a razor blade. The slices were then transferred to small plastic vials and I ml. of double glass distilled water added to each slice. The vials were stoppered and allowed to stand (at 4°C)

for one hour, with occasional agitation. The pH of each vial was then measured with the EIL 7030 Laboratory pH Meter (Electronic Instruments Ltd., Chertsey, Surrey, U.K.), calibrated for readings at 4°C, using the EIL 1140 200 micro-combination pH electrode (Electronic Instruments Ltd.)

Note: Beeley et al. (1972) determined iso-electric points immediately after completion of a run, using an aantimony electrode applied directly to the polyacrylamide gel. An excellent correlation between millivoltage and pH of liquid buffer solutions was found using the pH equipment described above. However in numerous trials where the electrode was placed on the polyacrylamide gel no consistent millivoltage readings were obtained, consequently resort had to be made to the less accurate method of determining the pH of individual slices.

Protein Staining Procedure

The fast staining procedure described in the Ortec Application Note AN 32 (1970) was employed.

The carrier ampholytes were removed by fixing the gel in 12.5% trichloroacetic acid at 65°C for 30 minutes. The gels were then stained in a 0.2% solution of Coomassie Brilliant Blue R.250 dye (G.T. Gurr Ltd.) in ethanol - acetic acid - water (45:10:45,V/V) for 30 minutes at 65°C. The gels were then destained in a solvent of ethanol-acetic acid-water (25:10:65,V/V) for 20 minutes at 65°C. This solvent was then changed and a further 30 mins. of destaining allowed at 65°C. The gels were subsequently cleared of background staining by several changes in 7% acetic acid at 65°C, before being stored at room temperature in 7% acetic acid.

Improvement of resolving power by admixing various pH ranges of Ampholine carrier ampholytes

Gel electrofocusing in urea was carried out in the initial experiments with ampholytes from a single batch covering the pH range 3.5-10. As described below in Chapter 4, this method provided a highly reproducible and sensitive technique for the separation of crystallin subunits but many of the major subunits focusing in the upper pH range of the ampholine gel were found extremely close together. Since accurate estimates of protein turnover require sharp DPM profiles and ratio-peaks that can be definitely ascribed to each polypeptide, it was obvious that in order to make maximum use of the gel electro-focusing technique improved resolution of many components was necessary, regardless of the eventual classification ascribed to a particular polypeptide. However sufficiently improved separation could not be obtained by simply increasing the length of the gel.

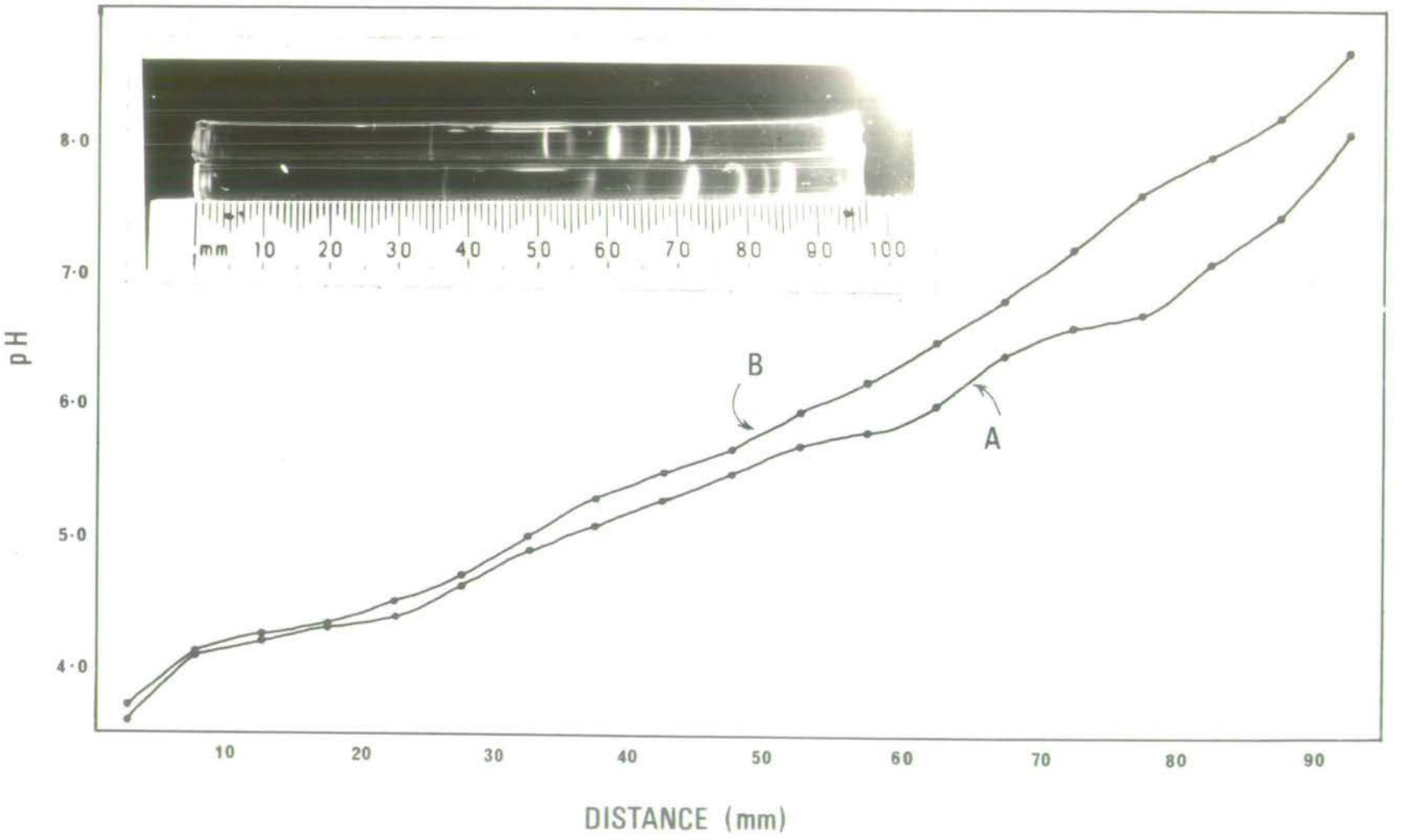
To obtain the maximum resolution available in electrofocusing the pH range should be as narrow as possible, i.e. just large enough to include the pI values of the proteins of interest (Instruction Manual, LKB 8100 Electrofocusing Equipment, LKB-Produkter AB, Stockholm-Bromma, Sweden). The ~~shallower~~ shallower the pH gradient is, the greater the separation distance is between any two proteins. But in order to maximally separate a series of components that have iso-electric points covering the pH range 3.5-10 it would be necessary to run each time a considerable number of gels containing ampholites of a narrow pH. The construction of a particular shape of pH gradient that would produce sufficient separation of crystallin subunits within the range pH 3.5-10 in a single, larger gel appeared to be a less tedious procedure, and more reliable, than

fractionating a protein sample on a series of gels. The recommendations of Haglund (1971) for work on pH ranges remote from the neutral point suggested that such a gradient could be constructed.

Haglund (1971) pointed out that when employing a pH gradient composed of carrier ampholytes with a pH in the acid range, say pH 3-5, the most basic species of ampholyte should concentrate at the cathode. However since water is the most basic ampholyte present in this system, it will tend to form a zone of pure water between the cathode and the carrier ampholytes. In this region the conductivity will drop to a very low level, and a large part of the potential drop will be dissipated in the water zone. Consequently other regions of the gradient may be subjected to a field strength too low to maintain the gradient or complete the transport of the components to focusing sites along the gradient. Furthermore the amount of heat dissipated in the water zone could conceivable ruin the whole experimental run. The author recommended supplementing the carrier ampholytes of the acid range with carrier ampholytes from the fraction pH 6-8. This addition bridges the gap between the cathode and the carrier ampholytes, no water zone is formed and a reasonable field strength can be obtained in all regions of the system. However the pH range, in this case, is linear in the acidic pH region of interest then the pH curve rises steeply due to the added fraction of carrier ampholytes pH 6-8. Similar considerations apply also to work carried out with pH gradients in the alkaline range.

These alterations in shape of the pH curve by the admixing of ampholines from different pH ranges suggested that a particular gradient could be produced that covered the pH range 3.5-10 but

Figure 1. Comparison of pH gradient curves obtained with ampholytes (A) of pH ranges 4-6,6-8, and 3.5-10 in equal proportions, (B) pH 3.5-10 alone. The two types of 6M-urea polyacrylamide gels were prepared and focused in the same run, without protein samples. After (approx.) 16 hours of electrofocusing, the gels were sliced and the pH values of eluates determined as described in the text. Note the shallower form of curve A, particularly in the higher pH regions, and the higher final value of curve B. Insert : comparison of electrofocusing pattern of adult chick crystallin samples (at 250 ug gel) obtained with gradient A (lower gel) and gradient B (upper gel). Gels were fixed in 10% trichloracetic acid, then photographed with side lighting.



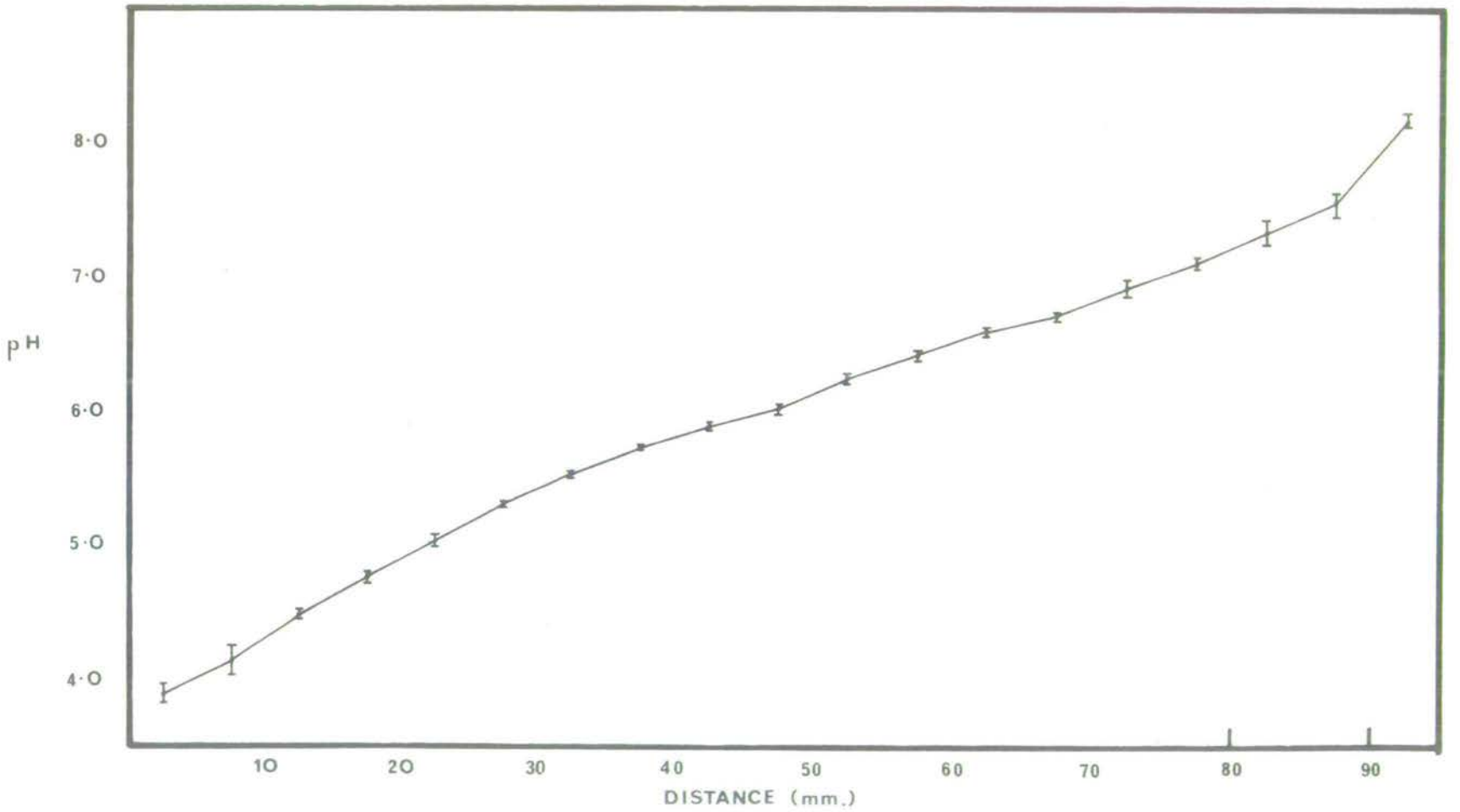
gave the greatest degree of resolution in the region where most of the components of interest were focused.

Preliminary studies with carrier ampholytes from narrow pH ranges indicated that many of the major subunits focussed in the pH range 6-8. Two considerations now influenced the design of a pH gradient that would maximally resolve the crystallin components over the whole length of a 10 cm. polyacrylamide gel, the largest size convenient for the electrofocusing tank. Obviously in order to resolve the maximum number of components, carrier ampholytes which cover the whole available pH range would be necessary. Secondly increased resolution in the pH range 6-8 would be a distinct advantage. Consequently equal amounts of ampholines from the ranges pH 3.5-10 and pH 6-8 were admixed together with a third equivalent of carrier ampholytes from a narrow pH range of either 4-6, 5-7, 6-8 or 7-10. Equivalent proportions from the ranges pH 3.5-10, pH 6-8 and pH 4-6 produced an overall pH gradient that gave excellent resolution of crystallin subunits over the whole length of the gel, as compared to the gradient formed from a single batch of ampholyte from the pH range 3.5-10 (fig.1). Considerably greater distances now separate the major subunits. This greatly enhanced resolution not only makes turnover studies of the major subunits much more feasible, it provides a technique where even very minor components can be investigated.

Reproducibility of Results

Minor differences in separation distances were noticed in different runs. These were probably mainly due to pipetting errors in admixing different pH ranges of ampholytes and differences in particular ampholyte batches. In general however a high degree

Figure 2. Reproducibility of pH gradient after iso-electric focusing in 7.5% polyacrylamide gel. Preparation of the gel and isoelectric focusing conditions were as described in the text using ampholytes in equal amounts from the nominal pH ranges 4-6, 6-8 and 3.5-10. After overnight electrofocusing, the gels were sliced and the pH values of eluates determined as described in the text. The graph represents the average gradient determined from four successive electrofocusing runs, the standard deviation from the average pH for each slice is represented by vertical bars.



of reproducibility in the shape of the final pH gradient could be maintained (fig.2).

Strained gels were shrunk in 50% methanol to their original length and the iso-electric points of the crystallin subunits read from the pH gradient curve obtained by slicing a duplicate, unstained gel run simultaneously. The iso-electric points of the components visible after fixation in 10% trichloracetic acid could be determined more directly, since the swelling of gels occurs in this solution. pI values of the crystallin subunits using ampholytes from the pH range 3-10 were in excellent agreement to those found from the shallow gradient where carrier ampholytes from the pH ranges (3.5-10, 4-6 and 6-8) were used in equal proportions (fig.1,3). These particular pI values are compared with other worker's findings, in the Discussion section, since subsequent chapters deal with the assignation of the major components to specific crystallin classes.

The use of urea solutions has become widespread in the elucidation of the subunit structure of proteins. However the possibility that multiple zones found in gel electrophoresis or gel electrofocusing may be artefacts arising from the use of urea as a dissociating agent must be taken into consideration (Stark et al.1960). These authors pointed out that cyanate formed during heating of urea can carbamylate free amino groups of proteins, thereby raising the net negative charge of the molecule. Consequently carbamylation would thus result in increased electrophoretic mobility of polypeptides towards the anode, whilst in gel electrofocusing the iso-electric points of the individual zones would be lowered. Indeed gel electrofocusing has been specifically

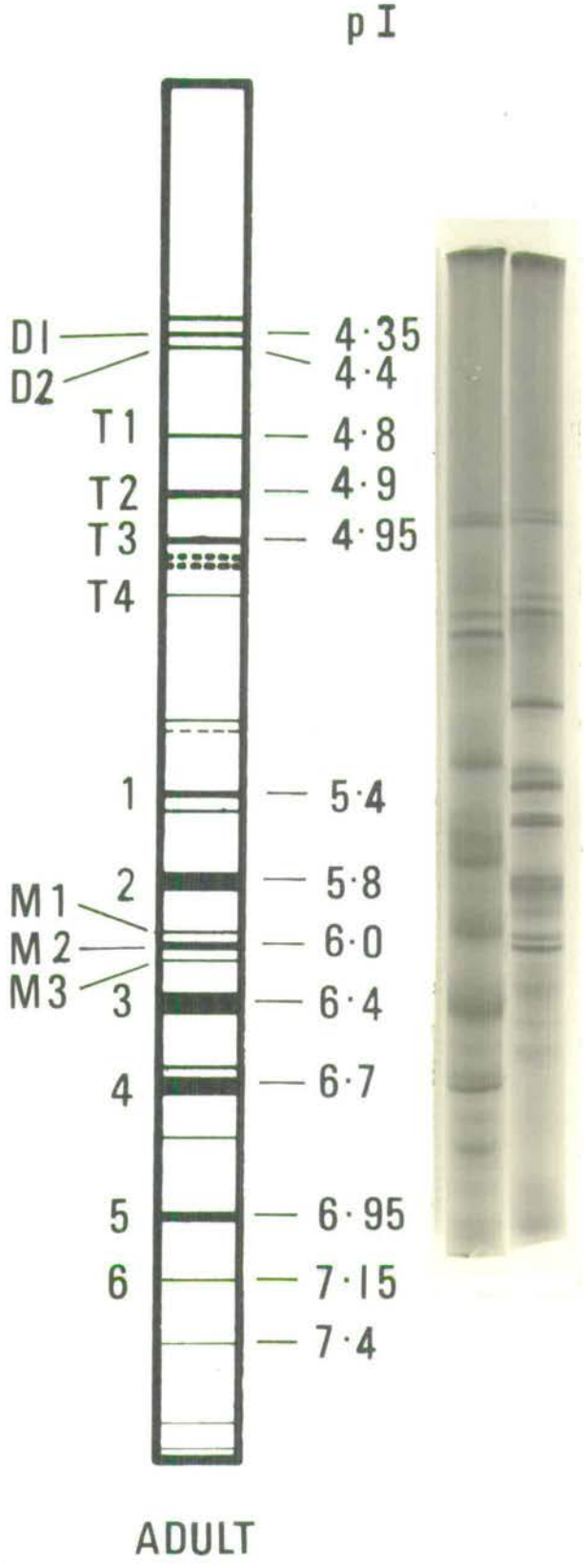


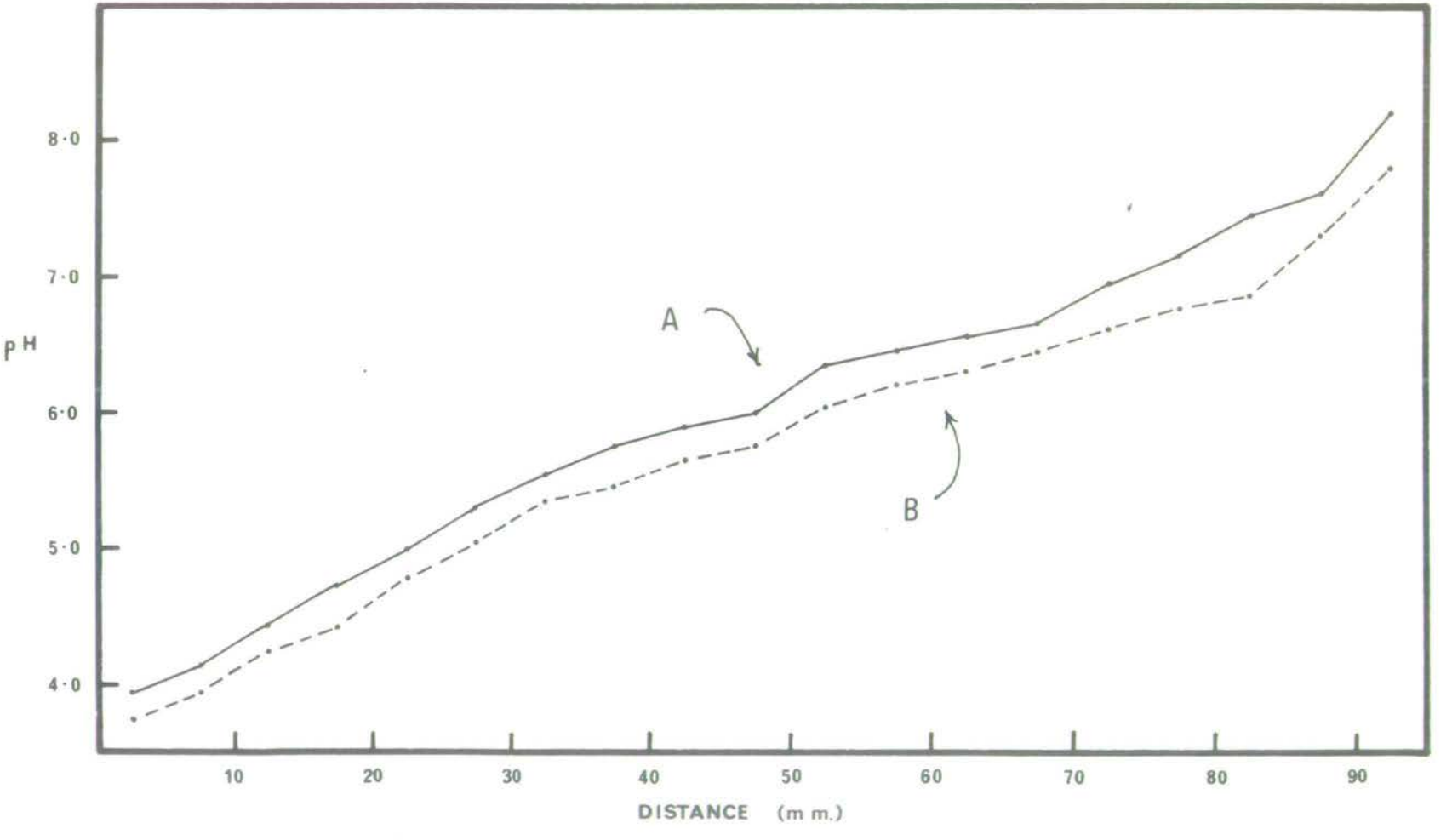
Figure 3. Diagrammatic representation of gel electrofocusing pattern of adult chick crystallins obtained with ampholytes of pH range 4-6, 6-8 and 3.5-10 in equal proportions, as revealed by Coomassie Brilliant Blue staining. Insert: comparison of electrofocusing obtained with this pH gradient(left) and ampholytes of pH 3.5-10 alone (right).

used to follow the successive carbamylations of amino-groups in chymo-trypsinogen A, after the protein had been heated for various lengths of time at 100°C in 8M urea (Bobb and Hofstee,1971).

However the formation of cyanate ions in urea solutions at low temperature is extremely slow (Hagel et al.1971). Furthermore attempts were made to keep the cyanate concentration as low as possible. Analar grade urea (B.D.H. or Fison) was used throughout and solutions were prepared by dissolving urea in cold water, for solutions above 6-molar only the minimum amount of warming was applied. Further purification of urea on a mixed ion-exchange column was omitted since Gerding et al.(1971) found that this step had little influence on the electrophoretic behaviour of bovine α-crystallin in 6M urea.

As shown repeatedly throughout this work, samples of chick lens crystallins dissolved in 8M urea and 100 mM 2-mercaptoethanol then applied immediately to ampholine gels containing 6M urea showed no difference in iso-electric pattern to samples dialysed for 24 hours against this solution, (in a tris-glycine buffer), prior to gel electrofocusing. That is, samples exposed to urea and 2-mercaptoethanol for 16 hours showed the same iso-electric pattern as those exposed for 40 hours, suggesting that under the particular conditions and times used carbamylation of proteins was not a major problem. It should be noted that the tris buffer employed in dialysis being a quaternary ammonium base, would tend to remove free cyanate ions from solution as they formed. Gerding et al. (1971) also found that samples of bovine α-crystallin dissolved in 6M urea and I mM DTT and applied immediately to a polyacrylamide gel gave identical electropheretic patterns to

Figure 4. Effect of 6M urea on the pH gradient in a polyacrylamide gel column after isoelectric focusing. Gels were prepared with and without 6M urea, by the procedure described in the text, containing ampholytes from the nominal pH ranges of 4-6, 6-8 and 3.5-10, in equal amounts. Gels were focused overnight without protein samples in the same run, then sectioned and the pH (at 4°C) of each 5mM slice measured (see text) : A) urea B) no urea



samples that were similarly dissolved and applied after 24 hours standing. Marked carbamylation effects were shown only after several days standing of the protein in 6M urea, in the absence of any buffers.

The effect of 6M urea on the iso-electric points of Ampholine carrier ampholytes after gel electrofocusing is shown in fig.4 In the absence of urea the gradient ranged from pH 3.75 to 7.8 and from pH 3.95 to 8.2 in the presence of urea at $4^{o}C$. Urea, on average, raised the value of the observed pH range by 0.3 pH unit, the increase ranging from .2 to .6 of a pH unit. A second comparison showed an average increase of 0.36 pH unit in the apparent iso-electric points of the carrier ampholytes in the presence of 6M urea.

These values are in good agreement with the results of Salaman and Williamson (1971) who found that 6M urea raised the observed pH value of carrier ampholytes by between 0.3 and 0.4 pH unit, using a similar method of measuring pH after soaking urea gels in water. Ui (1971 a,b) also showed that 6M urea raised the pI of carrier ampholytes by 0.42 pH unit.

Using a column iso-electric focusing method, Josephson et al. (1971) found that 7M urea raised the pI of the carrier ampholytes by about 0.9 pH unit throughout the pH range 3-6. This value may be more accurate since with this technique no dilution of samples with water is required for pH determination. Although this effect appears to be poorly understood, all of these authors agree that urea depresses the ionisation of certain groups contributing to the iso-electric point of the molecule. If ionisation is depressed,

basic side groups will retain protons and the rise in pH follows from the diminished concentration of H^+ ions in solution.

Josephson et al.(1971) make the important point that the effect of any cyanate formed from urea during electrofocusing (which would tend to lower the iso-electric points of proteins through carbamylation) would be of minimal significance compared to the considerable rises in pI of ampholytes, that occur through the spontaneous effect of urea.

Possible artefacts arising in gel electrofocusing.

Mention has already been made that artefacts can occur where the initial pH at the site of sample application exposes proteins to pH regions at which they are unstable (Lewin,1970 and Nord, Kellgren and Wadstrom, in preparation, cited in Wadstrom and Smyth, 1973). As pointed out above,placing the lens crystallin sample at various positions in the gel gave no differences in result to where the sample was applied directly at the anode, hence artifacts of this nature seem unlikely to account for the observed heterogeneity.

A more serious possibility is that the multiple bands revealed by iso-electrofocusing may be due to the binding of different numbers of carrier ampholytes. Ampholine-protein complexes have been reported for wool proteins (Frater,1970) and bovine serum albumin (Wallevik,1973). The latter author points out that the binding properties of bovine serum albumin may however be extreme. A number of investigations have established that if such complexes are formed they are probably very weak and reversible. Radioactively labeled Ampholine covering the pH range 3-10 could be completely separated from human serum albumin on Sephadex G-50 (Vesterberg,1969)

in contrast to the results of Wallevik (1973) with bovine serum albumin and labelled Ampholines from the pH range 4-6. High binding affinities of Ampholines might be restricted to certain pH ranges, and overlooked if only small amounts of Ampholine come into contact with milligram quantities of protein (Wallevik, 1973). Wellner and Hayes (1973) found that some radioactivity remained bound to the protein when L-amino acid oxidase was electrofocused with H^3- labeled Ampholines, even after gel filtration on a Sephadex G-100 column. The bulk of this radioactivity could be removed by dialysis however, suggesting that any binding was not irreversible. They suggested that some of the bound radioactivity may be accounted for by amino-acid contaminants found in the ampholyte batch. Possibly however even weak and reversible binding of ampholines to proteins might delay the development of the final electrofocusing pattern and give false indications of heterogeneity (Wallevik,1973).

Because of these reported differences in the binding properties of proteins, and the possibility of artefacts arising through such Ampholine-protein complexes, additional criteria are required to demonstrate that any indications of heterogeneity are genuine. Thus, protein components should re-run as single bands to their original iso-electric point upon re-electrofocusing, if no complexing with carrier ampholytes occur. This has been shown for components of myoglobin (Fawcett,1968) and bacterial asparaginases (Robinson,1972). The chick lens crystallins isolated by flat gel electrofocusing re-run as single components to their original iso-electric point (Brahma, personal communication). The amino-acid composition of chick δ-crystallin was extremely similar whether initially purified by gel electrofocusing alone or gel

electrofocusing followed by gel filtration (Piatigorsky et al. 1974), again suggesting that any binding of ampholines to the protein must be weak and reversible. There appears a far greater possibility of observing artefacts of apparent protein heterogeneity or alterations of binding properties of Ampholines with proteins that bind many low meolcular weight ligands such as substrate molecules or fatty acids, rather than non-enzymatic proteins like the crystallins. However it must be stressed that reproducible results of isc-electric focusing of proteins in polyacrylamide gels can only be obtained by strict adherence to specific experimental procedures. In particular distorted boundaries and skewed gradients can easily occur if the initial power settings are too high.

Chapter 4

THE RESOLVING POWER OF GEL ELECTROFOCUSING IN UREA WITH RESPECT TO CHICK CRYSTALLIN SAMPLES

If each different polypeptide chain is specified by a different structural gene then an accurate estimation of the number of different polypeptides in any one crystallin class will give an insight into the number of related genes active in the lens. Since in ontogenic studies one would also ideally like to describe the correlation between cellular differentiation and the development of individual polypeptide chains, it is clear that a highly resolving method of subunit analysis is essential. Polyacrylamide gel electrophoresis in dissociating conditions has been used in this laboratory to examine the subunit composition of the crystallins of the chick (Clayton, 1969) and to follow the course of development of the polypeptide chains (Truman et al. 1972 a). With the development of a gel electrofocusing technique in urea it was necessary to see whether its reproducibility and resolving power was an improvement on the gel electrophoretic technique.

In these early gel electrofocusing experiments protein samples were fractionated with Ampholine carrier ampholytes from the nominal pH range 3.5-10, there was no admixing of carrier ampholytes from narrow pH ranges.

Comparative analysis of polymer fractions initially separated by polyacrylamide electrophoresis in non-dissociative conditions.

Total lens protein (400 ug/per gel) from adult chicks were electrophoresed exactly according to the method Truman (1968) used, (see Materials and Methods section).

The major bands separated by polyacrylamide electrophoresis of whole chick lens preparations were then precipitated by soaking the gels in 80% saturated ammonium sulphate and slices were cut out and designated as described by Clayton (1969), (see Materials and Methods section). The proteins were then dissociated by dialysis overnight

5a

5b

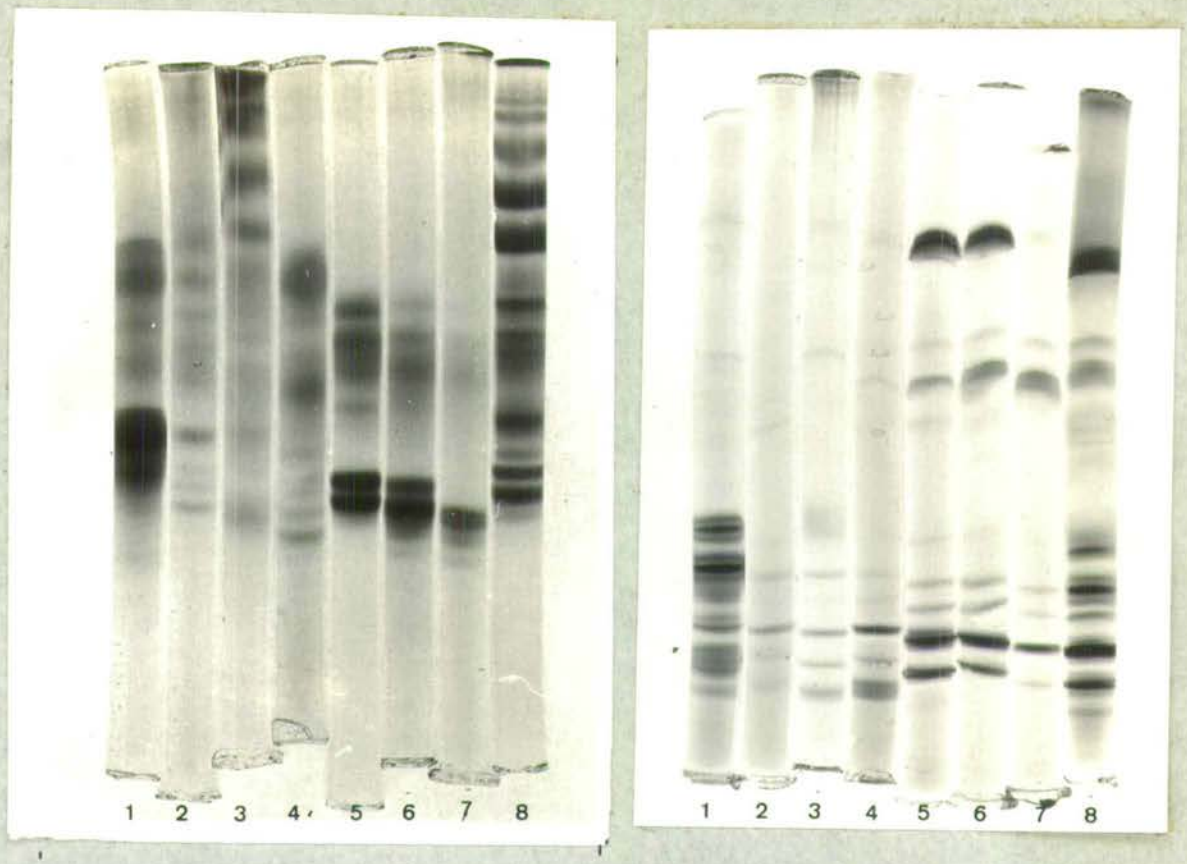


Figure 5. Comparative analysis of polymer fractions cut from polyacrylamide electrophoretic gels of total chick crystallins in the undissociated state. After electrophoresis the gels were soaked in 80% saturated ammonium sulphate and the protein precipitates excised, then dialysed overnight against 8M urea. Duplicate slices were then:

a) re-electrophoresed in polyacrylamide gel in the presence of 6M urea or b) electrofocused in polyacrylamide gel in the presence of 6M urea.

The samples were: 1) alpha. 2) intermediate alpha-delta.
3) delta. 4)intermediate beta.
5) fast beta-3. 6) fast beta-2.
7) fast beta-1. 8) total crystallin,(dissociated immediately before analysis).

against a buffer of 0.38 M glycine, o.38 M tris, pH 8.3 containing 8 M urea, 100 MM 2-mercaptoethanol, (The dialysis process also removed the ammonium sulphate from the polyacrylamide gel slices). Duplicate slices were then either re-electrophoresed into gels containing 6 M urea or analysed by isc-electric focusing in polyacrylamide gels also containing 6 M urea. In both cases a series of multiple bands was obtained in each gel, (Figures 5a and 5b).

Gels 5, 6, and 7 (figure 5a) designated as fast beta-crystallins in the schemata of Clayton (1969) show a distinct enrichment in two bands found near the anode in the total lens separation. Two slightly less anodal bands stain intensely in the gel re-run from the alpha region of the first separation procedure, although seven other bands are evident in gel 1 (figure 5a). Similarly the bands from the delta regions show a predominance in electrophoretic mobilities of a cathodal value, (see gels 2 and 3, figure 5a). Apart from such enrichments, however, the initial separation procedure on 7.5% polyacrylamide gels appears to have given fairly low resolution in this preparation. For example, the electrophoretic pattern of the delta region (gels 2 and 3, figure 5a) taken together contain all of the bands found in the preparation of total lens dissociated directly, (gel 8, figure 5a). A substantial number of components of the total electrophoretic spectrum can also be found in gel 1 (figure 5a), (alpha region) and gel 4 (figure 5a), (intermediate beta region). A more selective electrophoretic pattern is evident for protein fractions cut out from the anodal region of the original polyacrylamide gels (gels 5, 6 and 7, figure 5a). However if single protein bands are cut out from polyacrylamide gels in non-dissociating conditions (plain acrylamide) and are then re-electrophoresed into identical gels, further bands occasionally may be obtained (Clayton, 1969). Apparently the second electrophoresis may resolve the major

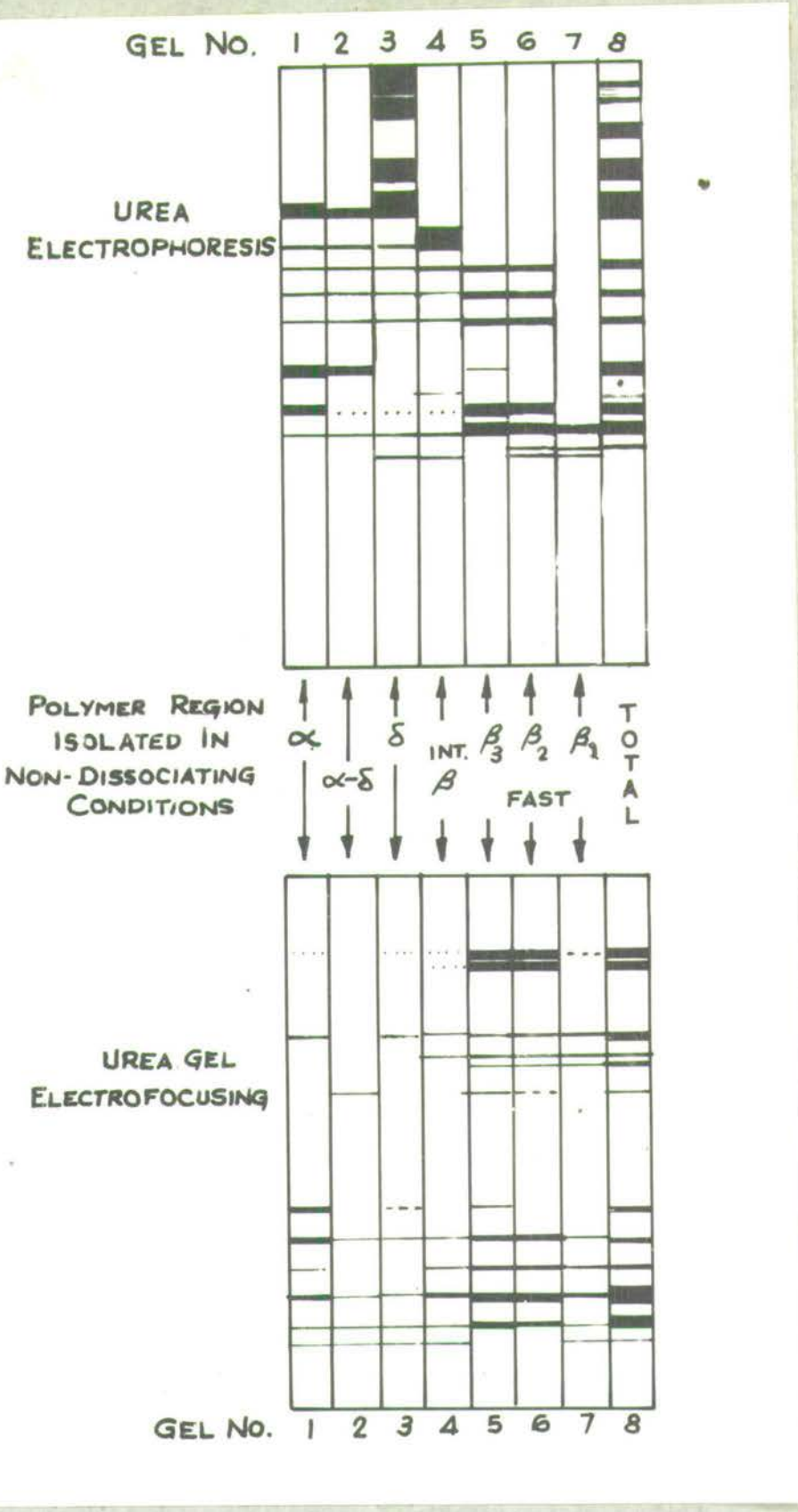


Figure 6. Comparison of the resolving power of urea gel electrophoresis and gel electrofocusing in urea, using polymer fractions obtained from the electrophoresis of total chick crystallins on polyacrylamide gels in non-dissociating conditions. The data is taken from fig. 5.

bands from several minor components. The multiplicity of bands observed is dissociating conditions for, say, the delta region (gels 2 and 3,figure 5a) may then be caused by contamination from other crystallin classes due to insufficient resolution in the first stage of plain acrylamide electrophoresis, although distinct enrichments of certain components can be observed. The electrofocusing results support these conclusions. For example, most of the protein bands obtained by iso-electric focusing of total lens crystallin in concentrated urea can be seen both in the alpha fraction (gel 1b, figure 5b) and the fastest beta region (gel 7b, figure 5b). All of the major proteins of high iso-electric point were common to the fractions, although their concentration varied with fraction. Again, enrichment of certain bands has occured. Two intense bands were apparent in the iso-electric spectrum of the alpha region (gel 1b figure 5b) whilst the various fast beta fractions (gels 5b, 6b, and 7b, figure 5b) also showed 3-4 bands that stained intensely in the intermediate range of iso-electric points. The subunit analysis of crystallin aggregates isolated from polyacrylamide gels in non-dissociating conditions by urea electrophoresis or iso-electric focusing in urea has been drawn up in diagrammatic fashion in figure 6 for ease of comparison. Numerous runs, in fact, established that comparison of protein bands from different samples and runs could be made unequivocably using the gel iso-electric focusing technique, whilst alignment of protein bands separated by urea electrophoresis was much more ambiguous and arbitrary. As well as providing greater reproducibility the iso-electric focusing method appeared more sensitive than the electrophoretic method of separation. For example in urea electrophoresis of the fast beta region (gel 5, figure 5a and 6a). Six bands can be observed whilst the sample can be separated into six major bands and six minor components by iso-electric focusing (gel5, figures 5b and 6b). This difference in resolving power suggested the possibility that a single protein band in urea polyacrylamide might show heterogeneity when analysed by gel iso-electric focusing in dissociating conditions. Since many investigations have assumed that a discrete band in urea

electrophoresis represents a single protein subunit, it was of considerable importance to establish whether or not this supposition could be supported by iso-electric analysis.

Conceivably the particular electrophoretic method employed in these studies might not be of sufficient power to separate subunits of very similar size and charge. Therefore it seemed that comparison of the protein complexity revealed by iso-electric focusing to that produced through urea electrophoresis would be of major interest. These studies were attempted before any assignation of subunits to particular crystallin classes and are described in the following section. However the initial results obtained with samples of crystallin aggregates isolated from polyacrylamide gels established the gel iso-electric focusing technique as a method of sufficient sensitivity and resolution to allow a highly detailed analysis of the crystallin subunits. The multiple bands that were found common to many crystallin aggregates, as revealed both by urea electrophoresis and iso-electric focusing techniques suggested that considerable purification of the undissociated protein fractions would be necessary before assigning any subunit to a particular crystallin class.

Comparison of the resolving power of urea gel electrophoresis and gel electrofocusing in urea

A comparison of the resolving power of urea electrophoresis and gel iso-electric focusing in dissociating conditions was made by isolating single protein bands obtained by the electrophoretic technique and subsequently subjecting them to iso-electric focusing in urea. Selected duplicate polyacrylamide slices were then further purified by a second electrophoresis in urea, cut out again and re-examined on ampholine gels.

In order to achieve maximum separation in the first urea electrophoretic run, the sample of total crystallin from the adult chicken lens was dissolved in 8 M urea, and electrophoresed into gels containing 6 M urea

for one further hour after the marker dye of bromophenol blue had left the gel. Thus the total time of electrophoresis was approximately 2¾ hours. The final separation was good, and a total of eleven bands and three interband regions could be clearly visualised when the gels were soaked in 80% saturated ammonium sulphate,(see figure 7a). These fractions were then dialysed against an 8 M urea buffered solution to remove the ammonium sulphate before being placed on ampholine gels containing 6 M urea. In addition selected duplicate slices were re-electrophoresed on 6 M urea gels, until the marker dye reached the bottom of the tube. The iso-electric spectra of the numbered urea slices is shown in figure 7b.

None of the urea polyacrylamide slices re-ran as a single band in the ampholine gels. Most of the slices in fact showed a remarkable degree of heterogeneity, certain fractions giving as many as six or seven components in the ampholine gels, although only two or three of these stain heavily, see for example gels 14 and 15 figure 7b. The electrophoretic mobility of the slices showed a general relationship to the iso-electric spectrum of its components. For example band 1, the fastest (anodal) region contained six components of low iso-electric point (see gel 2, figure 7b). The next most anodal band (gel 3, figure 7) has lost a component of low iso-electric point and consequently would be expected to travel slightly slower than band one, in the conditions described for electrophoresis at pH 8.9. As the electrophoretic mobility of the slices decrease a general gradient of increasing iso-electric point can be clearly discerned. Compare for example the iso-electric spectra of the fastest anodal band (gel 2, figure 7b) with that of the slowest, band 8 shown in gel 13, figure 7b.

Decrease of electrophoretic mobility can be seen to be accompanied by a decrease or loss of a component of low iso-electric point, or the increase in intensity or gain of a component of high iso-electric point. The electrophoretic mobility of a particular slice can be generally

Figure 7a. Electrophoresis of total chick crystallins on polyacrylamide gel in 6M urea. The diagram shows the position of the main bands after soaking in 80% saturated ammonium sulphate.

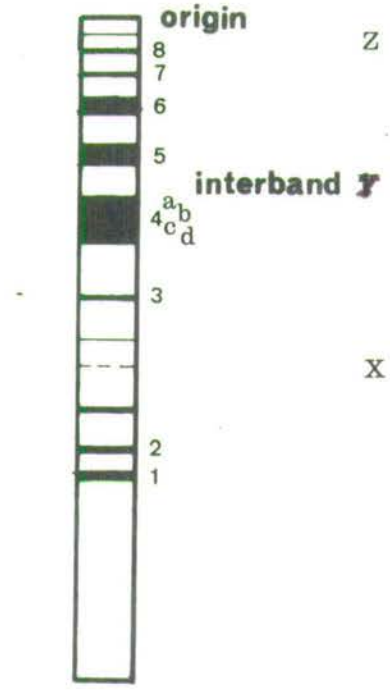
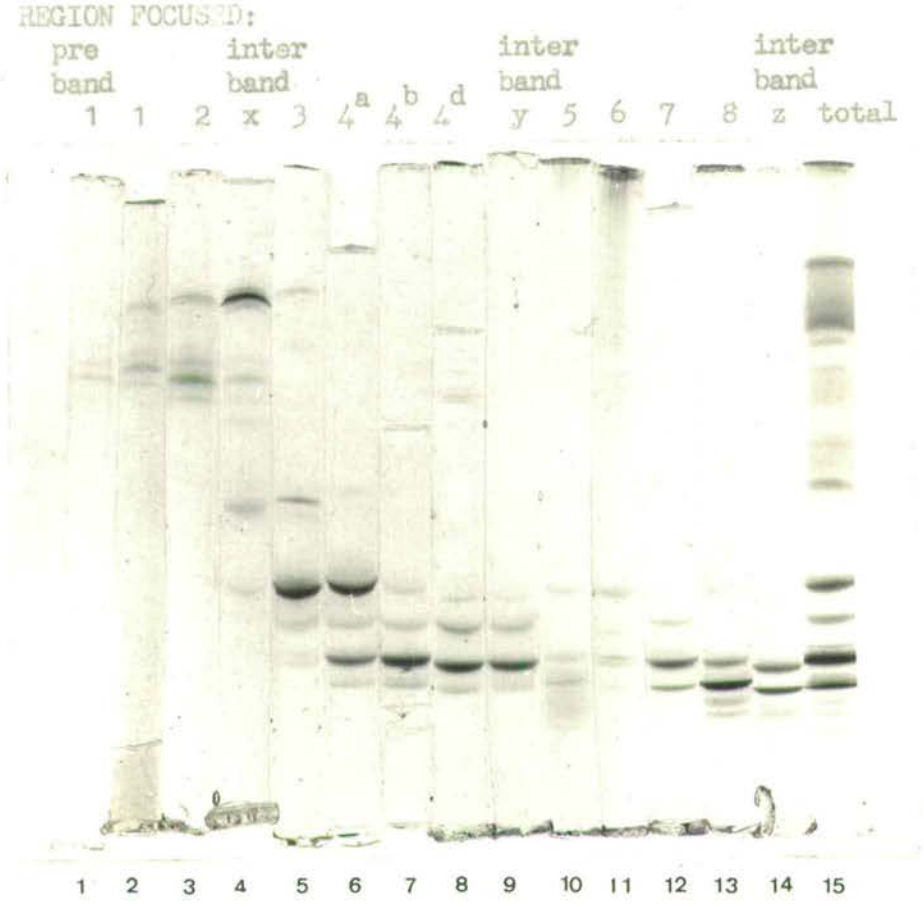


Figure 7b. Gel electrofocusing of regions obtained by urea polyacrylamide electrophoresis. The various regions designated in fig. 7a. were excised, dialysed free of ammonium sulphate, and analysed by gel electrofocusing in 6M urea.



correlated with the position and quantity of its most intensely staining components as determined by iso-electric focusing. None of the fractions analysed showed components additional to those found by gel electro-focusing of the total crystallins of the chick lens.

Experiments in which the urea polyacrylamide slices were purified by a second electrophoresis in urea provide further evidence that such slices may not necessarily represent a single component or protein subunit. Of the eight designated bands re-electrophoresed into gels containing 6 M urea, only three re-ran as single bands, whilst four other slices gave two separate bands. Apart from slice one, where the protein for some unknown reason did not electrophorese far into the gel, there appears the expected gradient of electrophoretic mobility between the different slices. Slice two for example representing the original band 2 ran much further to the anode than slice 8 (band 8), the slowest band analysed from the initial urea electrophoresis. The appearance of additional bands in the re-electrophoresis demonstrated that even separations in the high resolution technique of urea polyacrylamide electrophoresis are not necessarily unambiguous in the absence of additional criteria. Since separation in polyacrylamide gel is dependent on both the charge and molecular size of the protein samples, the molecular sieving effect of the gel is in fact selecting regions of similar charge density. Theoretically a large protein highly charged will migrate at the same rate as a relatively small protein which is only slightly charged. Thus identical electrophoretic mobilities may not always imply antigenic or structural similarities, even where the electrophoresis is carried out in dissociating conditions. A second separation procedure may reveal a remarkable degree of micro-heterogeneity. The analysis on ampholine gels of the highly purified slices obtained from the second urea electrophoresis substantiate this

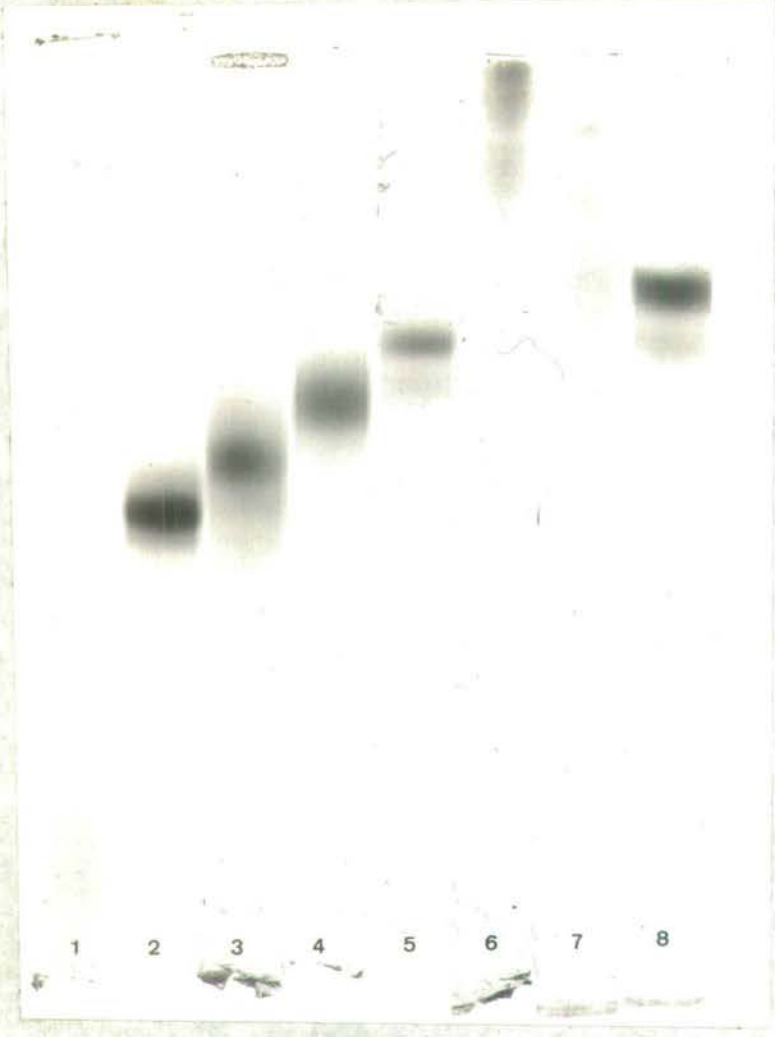


Figure 8. Results of re-electrophoresis on polyacrylamide gel containing 6 M urea of components isolated as single protein bands in the initial urea-polyacrylamide electrophoresis of total chick crystallins. The regions re-electrophoresed, designated according to the schemata of figure 7a, were, from left to right, bands 1,2,3,46,5,6,7, and 8.

conclusion. Duplicate gels to those shown in figure 8 were shrunk in 80% saturated ammonium sulphate, and slices excised and dialysed against 8 M urea - 2-mercaptoethanol, as previously described. All of the designated bands except band 6 were then analysed on ampholine gels in the usual manner. In the case of bands 2 and 8 it proved possible to cut out both major and minor bands that can be observed in the stained duplicates, in all other cases only the major band was excised.

All of the purified slices, save one, showed at least three components of separate iso-electric point (figure 9). Several slices, for example band 2 major (gel 3 in figure 9) and band 4b (gel 5 in figure 9) showed five components.

For ease of comparison, since these gels did not stain well, the comparison in resolution between the second urea electrophoretic run and the final iso-electric focusing run is presented in diagrammatic fashion in figure 10.

Again the electrophoretic mobility of a particular band appeared directly correlated to the iso-electric spectrum of its components, the slower bands containing proportionately more components of higher iso-electric points. If the iso-electric spectra of the purified protein bands are compared to the equivalent bands obtained from the first urea electrophoresis (figure 7a) it can be seen that although the major bands correlate exactly, several minor components of low iso-electric point have been removed by the second urea electrophoresis. The slices designated as bands 3 and 5 in the initial urea electrophoresis (gels 5 and 10 respectively in figure 7) have lost minor components through the second urea electrophoresis (gels 4 and 6 respectively in figure 9), although these particular bands are strongly represented in the fraction to major (gel 3 in figure 9). Whilst these results establish that the

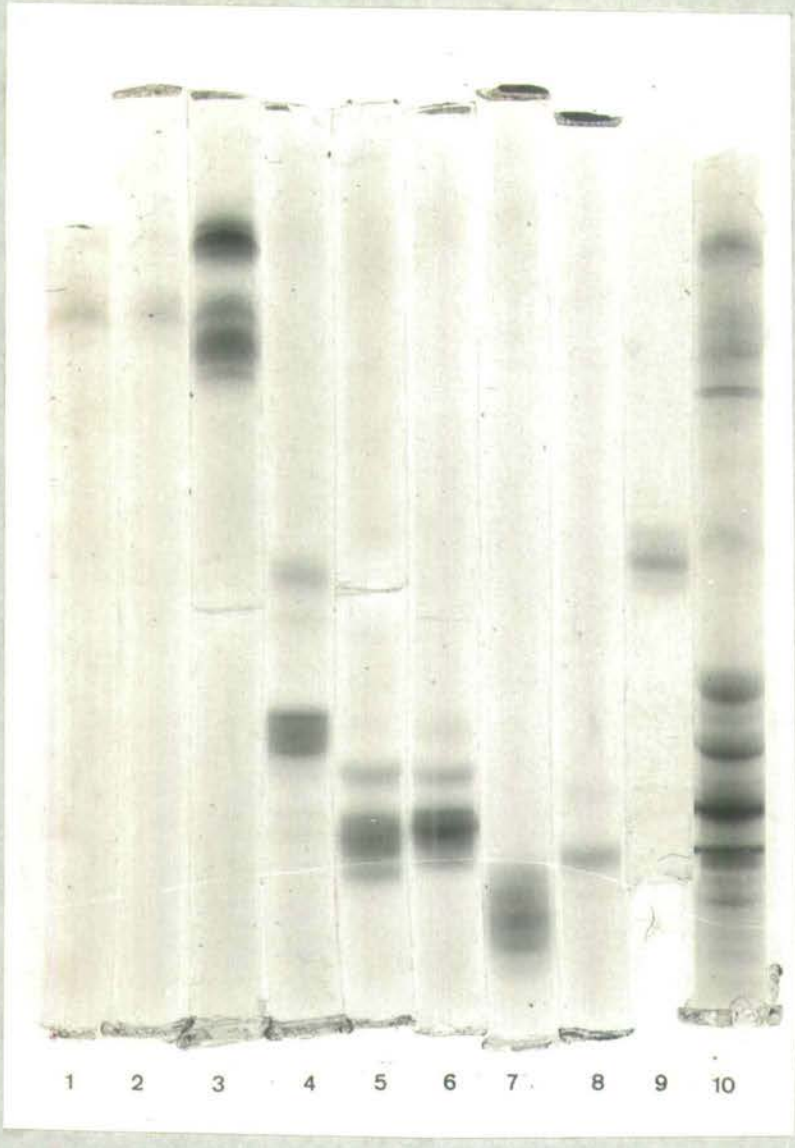


Figure 9. Results of gel electrofocusing of crystallin components prepared by double electrophoresis on polyacrylamide gel containing 6 M urea. Sampler :

1) Band 1
2) Band 2 minor
3) Band 2 major
4) Band 3
5) Band 4b
6) Band 5
7) Band 7
8) Band 8 major
9) Band 8 minor
10) Total chick crystallin

second electrophoresis had produced a further degree of purification of the samples, the heterogeneity of the samples was still distinctly expressed.

Clearly separation of the crystallins using a single procedure alone is rarely complete. Rather there is a considerable overlap between constituents of one fraction and another, both in dissociating and non-dissociating conditions. Since the polypeptide chains that comprise the aggregates in undissociated fractions fall within a range of similar sizes (Zwaan, 1968), it is to be expected that electrophoretic procedures may not give total separations. However the iso-electric focusing data assembled here suggests that even in dissociating conditions the total separations of such aggregative components of similar size and charge can not be achieved with this particular electrophoretic method. The increased resolution available with the iso-electric focusing technique indicates that a variety of polypeptide chains are co-electrophoresing, even in dissociating components.

Since the re-electrophoresis results also indicate that the fractions obtained by urea electrophoresis contain more than one component it is necessary to consider the implications of these findings on antigenic and antogenic studies where a single band in urea polyacrylamide has been taken to represent a single protein subunit. The re-interpretation of antigenic data is possible once the components isolated by gel iso-electric focusing have been assigned to a particular crystallin class. However in radio-activity studies, the level of isotope incorporation obtained from a single band in urea polyacrylamide will not represent the pattern of synthesis of a single crystallin subunit, since each fraction has in fact multiple components. Overlap of dissimilar components will bias the incorporation level towards the mean, whilst further complications arise from the presence of similar components in adjacent fractions, which will tend to reduce differences between adjacent slices.

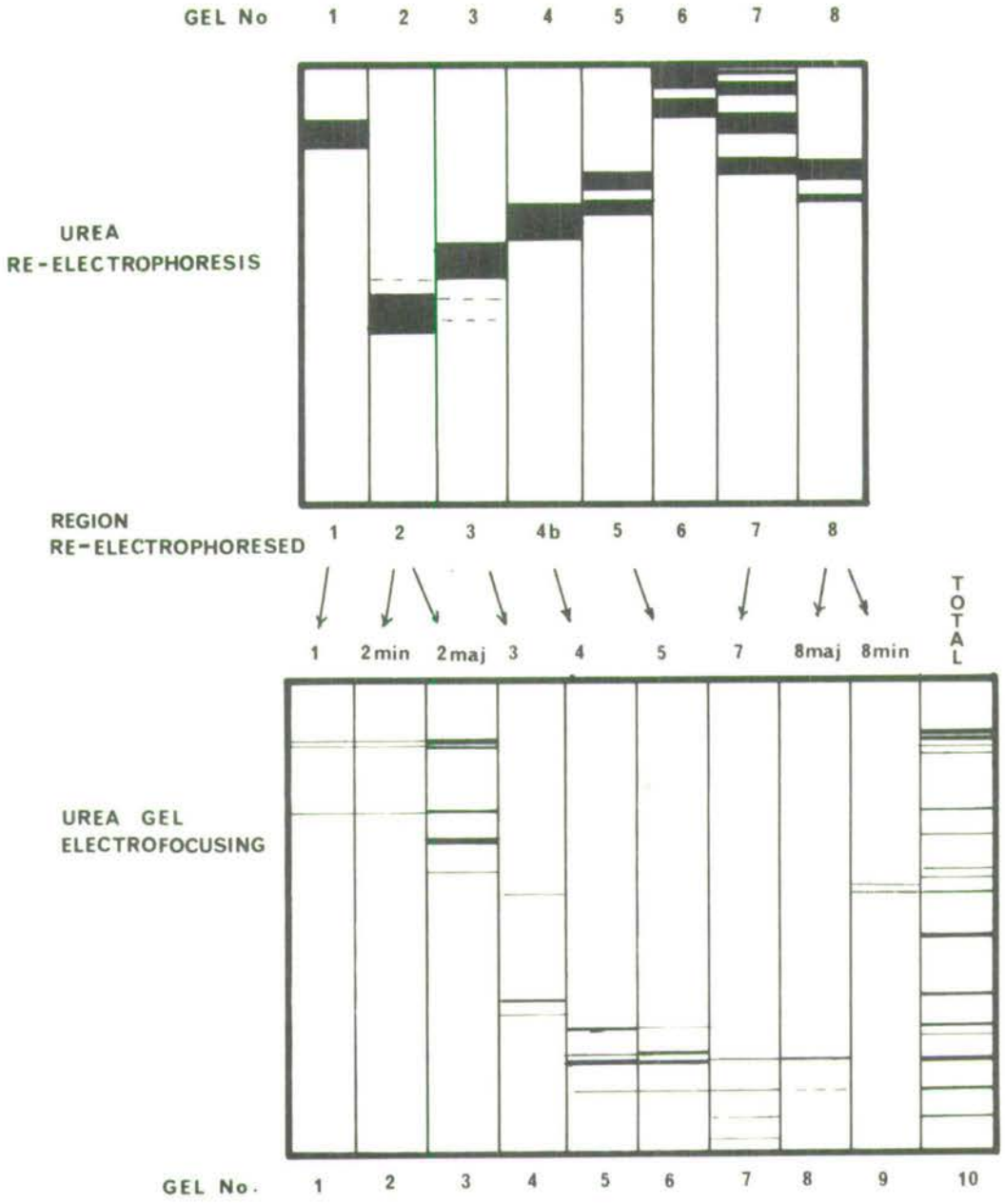


Fig.10 Diagram summarising the comparison of the resolving power of urea gel re-electrophoresis and gel electrofocusing in urea. Single protein bands obtained from the electrophoresis of total chick crystallins on polyacrylamide gels in dissociating conditions, were excised and further purified by a second electrophoresis in urea. Selected components were then cut out again and re-examined by urea gel electrofocusing. The data is taken from figs. 8 and 9.

If a particular component is being synthesised at a much faster rate than its associate components, the level of incorporation designated may reflect only the predominant component, and assignation of the correct pattern of synthesis to the more slowly made components will not be possible. The serious degree of overlapping of components in adjacent fractions, even after repeated electrophoresis, underlined the need to develop a highly resolving system that would separate dissociated components by a single parameter, even where the charge and size of the various polypeptide chains are very similar.

Three considerations influenced the rationale of further experiments at this time. Firstly the results described here indicated that multiple components could exist in a fraction that exhibits a single electrophoretic mobility during separation in urea polyacrylamide. Thus the difficulty of ascribing particular components to a particular crystallin class remained, even though a considerable degree of purification could be achieved through repeated electrophoresis in dissociating conditions. But identification of particular protein bands after shrinking urea polyacrylamide gels in 80% ammonium sulphate is considerably more difficult than the corresponding identification of aggregates electrophoresed in non-dissociating conditions. The order of appearance of precipitation bands and the pattern of solubility in ammonium sulphate are quite distinctive for several of the major aggregates (Clayton, 1969). Consequently precise identifications can be made after comparitively little experience of the precipitation technique. Most of the 10-14 bands that can be cut out of urea polyacrylamide gels after precipitation <u>in situ</u> show very similar properties of solubility, and identification requires great experience. Furthermore, fairly considerable variation was observed between urea gels electrophoresed in different runs although the repeatability within runs was quite good.

Other authors have reported similar findings (Day, 1971). The difficulty of comparing samples in urea gels electrophoresed in different runs, and also of precisely identifying the precipitated bands precluded the use of this technique as a purification procedure prior to further analysis by the iso-electric focusing technique. The purification of aggregates by a combination of different techniques, prior to subunit analysis, appeared a more feasible approach than purifying dissociated components. The description of these attempts to obtain comparatively pure crystallin aggregates and the results of the subsequent subunit analysis by iso-focusing in concentrated urea form the basis of the next section.

Further analysis of polymer fractions separated by polyacrylamide electrophoresis: Alteration of electrophoretic conditions to obtain maximal separation of particular components.

The results outlined earlier indicated that only purified or enriched crystallin fractions could be reliably used in any attempt at subunit classification. Selection of a separation method or combination of separation methods that might produce sufficiently pure fractions for sub-unit analysis appeared a formidable task. Whilst two major methods of resolution are available, based on differences in molecular weight or molecular charge of the sample components, ultimately any separation and classification would have to be correlated with either amino-acid sequences (e.g. the work of Croft, 1972 on bovine samples) or antigenic analysis. It appeared that the choice of suitable purification procedures or experimental approach to the problem of subunit classification might be aided by an exact comparison of aggregates separated on the basis of their electro-phoretic mobility, with their subunit composition as revealed by gel electrofocusing in dissociating conditions. Consequently electrophoretic conditions were chosed to enhance the resolution of particular aggregates, and the selected bands precipitated *in situ* with ammonium sulphate, cut out and stockpiled at -20°C prior to subunit analysis.

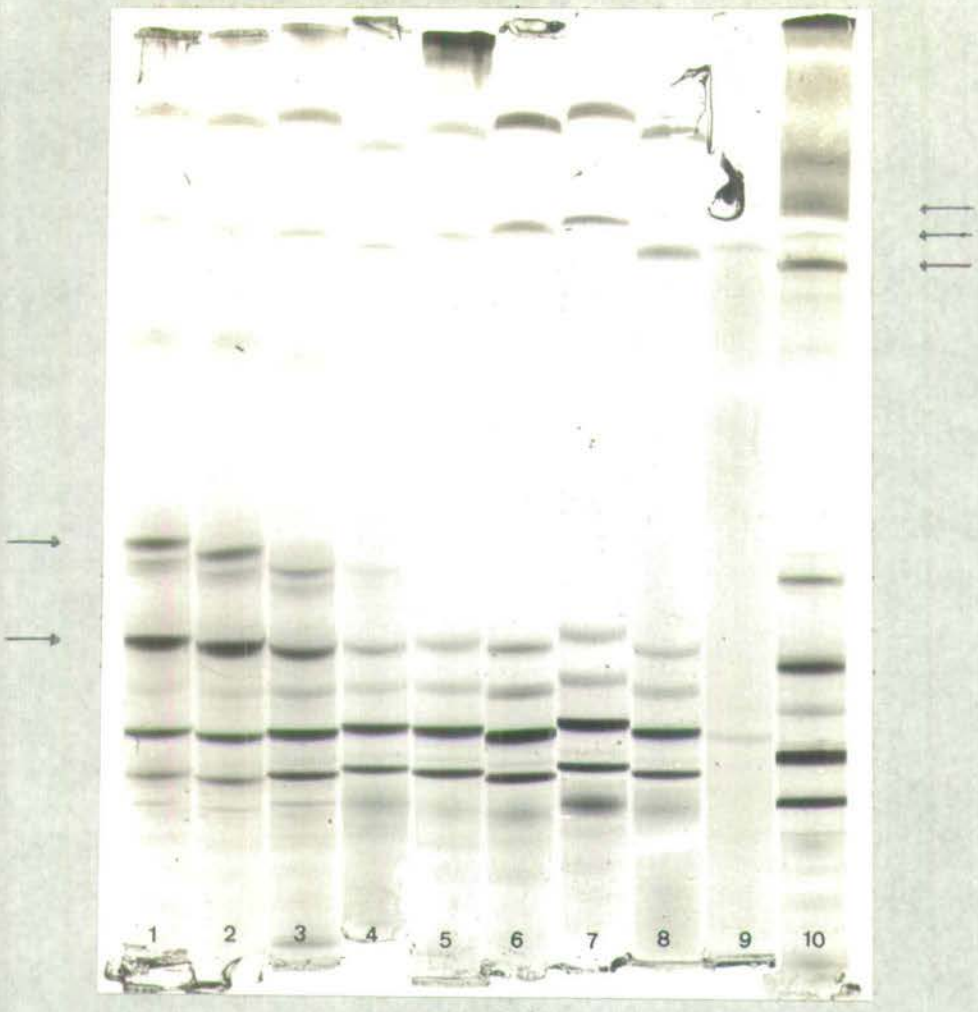


Figure 11. Urea gel electrofocusing of fractions obtained by the electrophoresis of chick crystallins on polyacrylamide gel in the absence of urea.

Samples: 1) - 4) progressively anodal fractions of alpha-crystallin, alpha 5, 4, 3 and 2.

5) delta.

6) intermediate beta-3 (slowest form).

7) intermediate beta-2.

8) intermediate beta-3.

9) fast beta.

10) total chick crystallin.

Since alpha-crystallins are the highest molecular weight proteins and show the lowest electrophoretic mobility in polyacrylamide gel, electrophoresis for a short period of time should favour the resolution of crystallins of faster mobility and reduce the degree of contamination by alpha-crystallins. If the marker dye of bromophenol blue was allowed to run two-thirds of the length of a gel, three components of high mobilty could be routinely observed and excised.

Similarly extended electrophoresis should remove the faster components, and favour the resolution and purification of the slower components. A maximum of five components could be resolved in the alpha-region, whilst three comparitively sharp bands could be excised from the intermediate beta region, if electrophoresis was continued for a further one hour after the marker dye had left the gel.

Although the characteristic appearance of the delta aggregate provided a reliable marker for all runs, each designation of a particular aggregate was based on the kind advice of Mrs. R. M. Clayton, who has worked extensively with this particular system, see Clayton, (1969).

The fractions stockpiled from the most successful electrophoretic separations were then dissociated by dialysis in urea and them analysed by gel electrofocusing in concentrated urea. The slices consequently represented the most highly resolved fractions that could be obtained by a single separation of chick crystallin under the described electrophoretic conditions.

The iso-electric spectra of each of the first nine designated fractions is shown in figure 11. These fractions were analysed together in a single run. Each fraction appeared to be composed of numerous components, with only a limited number of enriched bands corresponding to different regions of the electrophoretic separation. Numerous similar analyses were performed but the high degree of protein complexity and the

amount of overlapping of components throughout the whole electrophoretic mobility range was always observed. At least nine components could be found in all of the fractions separated by electrophoresis, even where conditions were chosen to maximise the resolution of particular aggregates.

Whilst the combination of the highly resolving technique of iso-electric focusing and the extremely sensitive stain Coomassie Blue has outlined the similarities in subunit composition of the various aggregates separated by electrophoresis, the high degree of overlapping and overall complexity of each of the aggregates poses difficulties in interpreting them as discrete physiological realities. The subtle gradations observed in the iso-electric spectra of various fractions suggest each aggregate represents an assembly of all of the major components with different proportions of individual components. The electrophoretic mobility of a particular complex, on this assumption, will be correlated with the iso-electric points of its major components. This interpretation implies that the components of the lens fibres when broken down by homogenisation then form complexes (perhaps randomly) involving all the major components, but in different proportions. Those assemblies containing a preponderance of components of low iso-electric point will have a high electrophoretic mobility in alkaline buffers, whilst the reverse is true for assemblies with components predominantly of high iso-electric point. If only slight differences in molecular charge occur between similarly proportioned complexes, the major degree of separation in polyacrylamide electrophoresis will be based on the molecular weight differences of the various aggregates. These speculations will depend obviously on the degree to which the type and number of a particular component can be interchanged to produce different complexes. The feasibility of this hypothesis is discussed, together with other interpretations, in a later section (see the Chapter 6) in the light of later experimental results.

A certain degree of enrichment of several components can be observed

however in figure 11 . Two strongly straining bands (arrowed) can be found in the slices designated as containing alpha-components, the intensity of the staining decreasing as the electrophoretic mobility of the aggregates increases. Similarly fast beta fractions are enriched in three components of low iso-electric point (see arrows in figure11), whilst comparatively deplete in the two components apparently characteristic of the alpha-fractions.

The most anodal band analysed (gel 9 in figure11) contained only four major components, three of low iso-electric point. Although the bands are quite faint, the fraction represents the lowest number of components, revealed by iso-electric focusing, isolated from an aggregate separated by electrophoresis. The enriched bands found in the alpha and fast beta regions are identical to those previously described in figure 5b. Compare the iso-electric spectra of the alpha region (gel 1) and that of the fast beta fraction (gel 7) as shown in figure 5b. These distinctive enrichments, consistently observed, indicated that a classification of subunits based on their iso-electric points would be possible provided suitably purified crystallin fractions could be obtained.

Analysis of polymer fractions initially separated by agar electrophoresis .

Since impure alpha fractions, isolated by electrophoresis, when analysed by gel electrofocusing in dissociating conditions showed a distinctive enrichment of at least two components, when compared to other aggregates, it was necessary to determine whether a similar iso-electric spectrum could be observed when alpha preparations were obtained by a completely different separation technique. Advantage was taken of the distinctive behaviour exhibited by alpha-crystallins when electrophoresed in agar, characteristically observed in immuno-electrophorestic studies. Indeed the most reliable evidence for the separation of crystallins into general classes is probably that of immunological specificity. It appeared possible that suitable agar separations might allow a direct correlation

to be made between antigenic specificity of aggregates and their subunit compositions, as determined by gel electrofocusing.

Preparations of total lens crystallins were tested by immuno-electrophoresis in agar at pH 8.9 (Campbell et al. 1968) as described fully in the Materials and Methods section. Figure 12a shows the immuno-electrophoretic pattern routinely obtained when such preparations were tested with an antiserum (designated 42W) against total chick crystallins.

In order to obtain sufficient material for subsequent subunit analysis ten microscope slides covered with 1.5% agar were employed in each electrophoretic run. Four wells per slide were cut out and 2 µl of sample at 80 mg./ml. were placed in each well. (The normal concentrations employed in this laboratory for immuno-electrophoresis, 2 µl of sample at 15 or 30 mg./ml. were insufficient for subunit analysis). Each sample was electrophoresed at 100 volts for two hours. Anti-serum was then placed in a trough cut 5 mm on each side of the well of the control sample. All other slides were then soaked in 80% saturated ammonium sulphate for one hour at 4°C. The precipitated bands were cut and designated as particular fractions with reference to the sample application point, that is the well. Either the major band between the well and the anode was cut out (total alpha) or further divided into portions such as leading, middle or trailing regions of alpha-crystallin. Five regions could be routinely identified by their precipitation pattern, alpha-middle, alpha-trailing, the region between alpha and the well (inter-alpha-well), delta (FISC) and a slow beta fraction.

Each region was cut in the form of an agar strip, dissociated in urea, concentrated and applied to electrofocusing gels, as described in the Materials and Methods section.

The excellent resolution obtained when the iso-electric spectra of the agar separations were analysed in dissociating conditions is shown in figure 12b. Each fraction exhibited multiple components. The

Figure 12a. Immunoelectrophoretic pattern obtained with total chick crystallins when tested with chick anti-total lens protein serum (42W). Both wells loaded with 2.5ul of adult chick protein at 15mg/ml. All other conditions were as described in the Materials and Methods section.

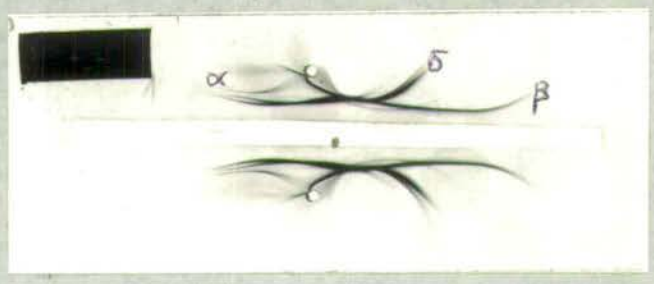


fig. 12a.

fig. 12b.

Figure 12b. Urea gel electrofocusing of impure alpha and delta crystallin fractions prepared by agar electrophoresis. Samples :

1) alpha - middle region of agar separation.
2) alpha - trailing region.
3) region between alpha region and sample well.
4) delta region.
5) slow beta.
6) alpha - total region of agar separation.
7) total chick crystallin.

total alpha (gel 6) and the alpha-middle fractions (gel 1) show three strong bands not present in the alpha-trailing fraction and depleted in the inter-alpha-well fraction and in the delta fraction (gel 4), (The delta fraction also contained two faint components of very high iso-electric point not found in any other fraction). The depletion of these major bands (arrowed) in the alpha-trailing and inter-alpha-well fractions suggested that these components have migrated significantly to the left of the well during the agar electrophoresis. However the fact that these components also migrate towards the cathode is shown by their strong representation in the slow beta fraction. These results emphasise again the considerable degree of everlapping of components in adjacent regions, although this is not to be unexpected since agar electrophoresis has considerably less resolving power than separation by polyacrylamide electrophoresis.

But the presence of so many apparently identical components throughout all the regions of the agar electrophoresis was perplexing, in view of the very discreet immunological arcs obtained for these self-same regions. Preparations of crystallins (from agar electrophoresis) that can be expected by immunological critera to be at least enriched in one particular class of crystallin appeared on the basis of gel electro-focusing to be composed of comparable subunits in different proportions.

However it should be noted that the two major enriched bands from the alpha region in agar are those also found in alpha-aggregates separated by polyacrylamide electrophoresis. The slow beta region in agar was also enriched in the four components consistently found (together with other components) in the polyacrylamide preparations of anodal aggregates. Furthermore alpha-enriched preparations could be partially separated from those fractions that appeared under the delta immunological arc. Thus separation by agar electrophoresis appeared to be of value as an initial purificatory technique to yield alpha and delta enriched fractions. Thus some subfractions of crystallin isolated from the same precipitin arc region show physiochemical heterogeneity

yet immunological homogeneity. Conversely many of the subunit components appeared to be identical with respect to physiochemical criteria such as iso-electric point and yet found in separate fractions as determined by immunological methods. Discussion of why immuno-electrophoresis gives less precipitin lines than the number of fractions that can be demonstrated by separation techniques is deferred at this point since more detailed experimental data is presented in a later section. However one finding is pertinent at this stage. A considerable degree of micro-heterogeneity of alpha-crystallin in agar gel electrophoresis has been observed (Zwaan, 1968). Furthermore two-dimensional polyacrylamide electrophoresis split the alpha-crystallin into three bands, and their elongated and curved shape suggested that aggregation and de-aggregation phenomena produced size heterogeneity even within the three main fractions. The author concludes that different degrees of aggregation together with slight differences in subunit composition may well account for this observed heterogeneity in size and the micro-heterogeneity revealed by immuno-electrophoresis. The subunit analysis obtained by gel electrofocusing revealed in addition that identical components may be found under separate immunological arcs. In general after agar electrophoresis chick lens gives basically three main precipitin bands with total crystallin anti-sera, yet a subunit analysis of various electrophoretic regions showed a comparatively poor fractionation of chick crystallins.

It might be valuable to summarise at this ppint the conclusions reached through the application of the analytical technique of gel electrofocusing in concentrated urea in investigation of the subunit complexity of the chick lens protein. The number of crystallins demonstrable in the chicken lens by polyacrylamide electrophoresis is much larger than the number of fractions revealed as separate precipitin lines by immuno-electrophoresis (Zwaan, 1968; Maisel and Goodman, 1965).

Many of the bands obtained by electrophoresis appear to be related polymers of varying composition (Clayton, 1969). Heteropolymer aggregates built up from the same combination of subunits but in different proportions could produce complexes of different electrophoretic mobility. When these well characterised aggregates were analysed both by electrofocusing or urea electrophoresis, the results described here revealed that components showed a remarkable degree of overlap from one fraction to another. Even where electrophoretic running times were varied to obtain maximal separation of particular aggregates, a complex iso-electric spectrum was still observed. However the separation of lens proteins by column chromatography and elution by discontinuous buffer systems to give several fractions has been analysed by immunological methods. Extensive overlap of the constituents from one fraction to another has observed (van Doormaalen et al. 1964; Zouzias and Manski, 1972). Consequently it appears that no single separation technique can completely resolve crystallin aggregates. It is indeed doubtful whether any of the aggregates analysed here represent assemblies of protein subunits that can be found in vivo. But ultrastructural studies employing highly specific anti-sera directed against particular subunits may indicate the inter-relationship of different crystallin classes in molecular structures that exist naturally with the lens.

The difficulties of resolving complexes consisting of peptide chains of similar sizes were further underlined by a comparison of the separations obtained by urea polyacrylamide electrophoresis and gel electrofocusing in urea. Single bands isolated after two electrophoretic runs in urea still showed consideralbe heterogeneity when analysed on ampholine gels. These results, taken together with the observation that re-running slices into a second urea gel may produce several bands, indicated that urea electrophoresis may not always be capable of resolving single polypeptide chains. Apparently even in dissociating conditions co-electrophoresis of very similar polypeptides can take place. However a considerable degree of purification of crystallins could be obtained by

re-electrophoresis in urea, and the electrophoretic mobility of a particular band appeared to be a simple function of the iso-electric points of its components.

Constituents of different fractions isolated by agar electrophoresis, a separation made mainly on the basis of molecular charge, also showed considerable overlap from one fraction to another, when analysed by gel electrofocusing in urea. This finding again emphasised the remarkable ability of all of the monomers to enter into the formation of aggregates. The pattern of enrichment of certain components in different agar fractions was gratifyingly similar to those found in immmnologically related fractions isolated by electrophoresis.

Chapter 5

IDENTIFICATION OF SUBUNIT COMPONENTS REVEALED BY GEL ELECTROFOCUSING

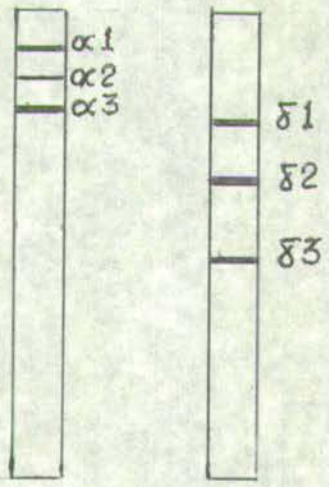
Preliminary studies with partially purified samples of chick alpha and delta-crystallins.

The development of a gel electrofocusing technique of improved resolution suitable for the accurate determination of the specific activities of the different subunits (Chapter 3) also enhanced the possibilities of accurately classifying crystallin subunits on the basis of their iso-electric point. The simplest, approach to this problem appeared to be in achieving at least a partial separation of the alpha, beta and delta-crystallins and re-running such enriched fractions on iso-electric focusing gels. However the difficulty of fractionating the proteins of the chick lens into major, uncontaminated classes through polyacrylamide or agar electrophoresis has been outlined in previous sections. Moreover similar difficulties in fractionating the components of the chick lens by gel filtration have been described (van Doorenmaalen et al. 1964; Truman, 1968) although the application of these techniques proved successful with the resolution of bovine lens proteins (Bjork, 1961, 1964; Testa et al. 1965). An improved separation can be obtained by using a sequence of three types of gel media (Truman, 1968). Nevertheless the author emphasises that a single passage through any system of gel columns does not separate chick lens proteins into three crystallin classes. This can only be partially achieved by re-running selected fractions through gel columns in order to decrease contamination by other protein fractions. The partial separations available by this preparative procedure appeared to be suitable for subsequent classification of chick lens proteins. Two preparations obtained by a single passage through the system of columns described in Truman (1968), were the gift of Dr. D. E. S. Truman of this laboratory. Immuno-electrophoresis revealed one fraction to be enriched in alpha-crystallin components, whilst the other

was similarly enriched in the delta-crystallin fraction, although the degree of enrichment was comparatively slight. Two other samples obtained be repeated re-running on the gel columns appeared to be immunologically pure preparations of alpha and delta-crystallin respectively, and these were also donated by Dr. Truman at later dates.

The partially purified fractions were re-run on polyacrylamide gels prior to subsequent subunit analysis. A spacer gel of 3.5% polyacrylamide, with an acrylamide/bis monomer ration of 37:1, made up in the usual Tris-glycine buffer, was employed in order to retain all starting material. All other electrophoretic conditions were as described in the Materials and Methods section, and the proteins were electrophoresed until the marker of bromophenol blue reached the end of the gel. The proteins were then precipitated in situ by shrinking the gels in an 80% saturated solution of ammonium sulphate (Clayton, 1969). The results obtained are summarised in figure 13a. The partially purified preparation of delta crystallin from adult lenses ran as three major components, two intermediate electrophoretic mobility, and one component of high electrophoretic mobility. Three bands could also be detected after ammonium sulphate precipitation in situ of the proteins electrophoresed from the impure alpha-crystallin fraction. Two discrete components showed a low electrophoretic mobility, and the most diffuse component electrophoresed just behind the slowest component of the enriched delta-crystallin sample. The six major bands described, together with the two spacer gels, were then excised from the plain gels, and processed in the usual manner for subunit analysis. The results obtained by gel electrofocusing the slices in 6 M urea are shown in figure 13b. Both preparations show considerable microheterogeneity. Most of the components resolved by iso-electric focusing could be found in all fractions, although different patterns of enrichment were obvious. The overlap of the constituents from one fraction to another, and their similarity to that of the total crystallin preparation, suggest that only a poor separation had been obtained in the initial separation by gel

Figure 13a. Diagram representing electrophoretic pattern of impure chick crystallin fractions prepared by gel filtration. Left: alpha fraction, Right: delta fraction.



13a

13b

Figure 13b. Urea gel electrofocusing of alpha and delta fractions obtained by gel filtration and further purified by electrophoresis on 7.5% polyacrylamide gel, in the absence of urea. A 3.5% polyacrylamide spacer gel was also employed in this separation. Samples: 1) Total chick crystallin 2) spacer gel region of alpha sample 3) alpha-1 (slowest) 4) alpha-2 5) alpha-3 6) spacer gel region of delta sample 7) delta-1 (slowest) 8) delta-2 9) delta-3.

filtration. Although there is a difference in enrichment pattern between the two fractions of impure alpha and impure delta-crystallins, electrophoretically separated components of the same sample show considerable similarities in staining pattern. However since the dye employed here, Coomassie Brilliant Blue R250, deviates notably from Beer's law at high protein concentrations (Chrambach et al. 1967), small quantitative shifts in protein components from one fraction to another may not be detected.

The iso-electric spectra of the two major fractions suggest that the major degree of resolution obtained in the electrophoresis was obtained on the basis of the molecular weight of the aggregates, rather than molecular charge. Thus the alpha-crystallin fractions utilised here, in general, contain components of lower iso-electric point than those of the impure delta-crystallin, and consequently should be more highly charged at pH 8.9. Yet the delta-crystallin fraction shows uniformly a higher electrophoretic mobility, in polyacrylamide gels, presumably because of their lower molecular weight. Since the staining patterns of all the electrophoretic fractions of the alpha-crystallin samples are similar, yet differ considerably from those of the delta-crystallin sample, the evidence suggests that within one sample, the electrophoretic fractions while being quite different in molecular weight, are composed to a great extent of similar polypeptide chains. Possibly the faster electrophoretic bands are composed of similar polypeptide chains in the same proportions as those of the slower bands, but in a lower degree of aggregation, and consequently less retarded by the molecular sieving effect of the polyacrylamide gel. This possibility could be investigated by highly accurate evaluations of protein concentrations within different electrophoretic fractions, through quantitative densitometry, using a quantitative stain such as Fast Green (Gorovsky et al. 1969).

These considerations apart, distinct patterns of enrichment were

obtained from the two samples. The sample enriched in alpha-crystallin showed nineteen to twenty-one components in the ampholine gels, and sixteen of these components were common to all three electrophoretic separations. The two fastest electrophoretic regions had nineteen components in common. The major feature of the iso-electric spectrum of each of the electrophoretic separations is, however, the presence of three very strongly staining components of intermediate iso-electric point, none of which are strongly represented in the delta-crystallin separations. The alpha spacer gel revealed no components in the electrofocusing run, suggesting that all of the protein sample had entered the running gel.

Each of the delta-crystallin enriched fractions showed twenty-one components in iso-electric focusing gels, of which seventeen were common to all separations. The delta-crystallin fractions, in general, showed components of both high and low iso-electric points that stained more strongly than those of the alpha-crystallin sample. The region of intermediate iso-electric point was depleted, however, in comparison to that of the alpha-crystallin fractions. Two very strongly staining components of higher iso-electric point were common to all of the delta-crystallin fractions. Interestingly, the delta-crystallin spacer gel produced two weakly staining components of identical iso-electric point to the two major bands apparent in the alpha-crystallin fractions. Since alpha-crystallin is characterised as the fraction of highest molecular weight, it would be expected under these electrophoretic conditions to enter into the running gel behind the other major crystallin classes. In the separation described here, a fraction of apparently alpha-crystallin containing two components has been retained in the spacer gel. Since similar components were not retained in the alpha spacer gel, it appears that the composition of the aggregates entering the gel may depend on the relative proportion

of each of the subunits available in the sample. A preponderance or enrichment of a particular type of monomer might produce a novel range of aggregates differing in size and composition. Very little is known either about the types of interactions between molecules of within one class of protein and interactions between members of the different crystallin classes.

The two most strongly staining bands enriched in the alpha-crystallin fractions were identical to those obtained from polyacrylamide electrophoretic separations of high molecular weight crystallin, and the alpha regions cut out from agar electrophoretic plates, described in the previous chapter. Furthermore two bands could be tentatively described as belonging to the delta-crystallin class from the pattern of enrichment obtained with the impure delta-crystallin fractions.

The main value of the gel filtration method is in its usefulness as a first step in the separation of the chick lens proteins according to their molecular dimensions. The poor degree of separation obtained by a single passage through a gel column system, as judged by the highly resolving technique of iso-electric focusing, emphasised again the difficulty of fractionating the chick crystallins. This has been suggested to be due to a polydispersity of their molecular weights, so that there is a virtually continuous range of aggregates present (Truman, 1968). Presumably this produces a similar range of molecular dimensions within aggregates. However even in successful fractionations of bovine lens, by gel filtration, several protein components were revealed by immunological techniques to be present in all fractions (Testa et al. 1965). The authors attributed this finding to the denaturation and subsequent aggregations of some components, at high hydrogen ion concentration.

The high resolution obtainable by gel electrofocusing revealed many components to be in common between the two partially purified samples.

Since relatively few subunits could be even tentatively ascribed to a crystallin class, it was obvious that definite classification of further subunits would await the production of much more highly purified samples. In the meantime it was decided to try and obtain increased resolution of protein aggregates by subjecting them to re-electrophoresis on polyacrylamide gels with an increased degree of cross-linkage between the acrylamide and bis monomer.

Improved resolution by re-electrophoresis into polyacrylamide gels of increased cross-linkage

The mobility of bovine alpha-crystallin is markedly affected by the degree of cross-linkage in the polyacrylamide gel (Jongkind et al. 1962). Recent studies stress that the degree of cross-linkage may have a remarkable influence on electrophoretic migration, perhaps by first affecting the pore size or in some yet undetermined manner (reviewed in Chranbach and Rodbard, 1970). Increasing the total concentration of acrylamide to obtain greater resolution was not feasible since components of the alpha-crystallin and delta-crystallin classes do not penetrate 8% gel (Zwaan, 1968). The degree of overlap of identical components between different aggregates as detected by iso-electric analysis, precluded excluding high molecular weight components out of firmer gels, since it could not be assumed that the smaller components entering the firmer gel were exclusively of one class.

Increasing the degree of cross-linkage slightly, seemed likely to take advantage of any increased resolution whilst at the same time ensuring all components entered the gel. Subsequently the degree of cross-linkage was altered from 2.6% (acrylamide / bis ratio of 37:1) to 3.5% (acrylamide / bis ratio of 27:1) in all gels for re-electrophoresis.

Proteins were electrophoresed at a concentration of 400 µg/per gel in the usual manner in gels with 2.6% cross-linkage as usual. The proteins

Figure 14. Urea gel electrofocusing of alpha and delta fractions obtained by gel filtration, then twice purified by electrophoresis on polyacrylamide gels, in the absence of urea. The bis/acrylamide ratio of the first electrophoresis was 37.1, and 27:1 in the second run.

Samples: 1) Total chick crystallin.

2) alpha (unpurified from column).

3) alpha (after re-electrophoresis).

4) major fraction delta (after re-electrophoresis).

5) and 6) fastest and slowest component respectively (A and B) of intermediate delta fraction.

7) and 8) fastest and slowest component respectively (C and D) of fastest delta fraction.

were then precipitated *in situ* with ammonium sulphate and the major bands excised and dialysed for 24 hours against Tris-glycine buffer and then re-electrophoresed into gels of 3.5% cross-linkage. Four major bands were obtained in the first separation. The position of the precipitated bands was marked in a control gel by tiny pieces of glass tubing, and the control gel stained with amido-black in the normal manner. The four fractions cut out were designated as alpha-crystallin, the main delta band and two faster delta aggregates, according to the usual classification developed in this laboratory (Clayton, 1969; Truman et al. 1971).

Upon re-electrophoresis in the modified running gels the alpha and delta-crystallin could be isolated as very sharp regions, after precipitation in ammonium sulphate. The two faster delta regions both split into twp bands in the gels with an increased degree of cross-linkage. All six electrophoretic regions were excised and processed for subunit analysis as normal, together with a preparation of alpha-crystallin obtained by repeated gel filtration. The result of this analysis is presented in figure 14.

The alpha-crystallin preparation prepared by gel filtration showed an iso-electric spectrum very similar to that of the alpha-crystallin obtained from repeated electrophoresis, and the same two major enriched bands described for the previous run (figure 13b) were obtained. The alpha-crystallin fraction obtained electrophoretically, in fact, contained slightly fewer components suggesting the double electrophoresis technique had considerable resolving power. However a third band (arrowed) strongly represented in the previous figure 13 was not strongly represented in either of the alpha-crystallin fractions.

The delta-crystallin fraction appeared to be the purest sample analysed up to this time. The three most strongly staining bands were components of high iso-electric point, and included both major components

found in the enriched delta-crystallin fractions whose analysis was described in the previous section (see figure 13b). The two major bands found with all alpha-crystallin preparations (designated components 1 and 2) were also present, but stained far less intensly than the corresponding alpha-crystallin fractions. A few very faint bands were detected at the very top of the ampholine gel, a feature that occured occassionally with other delta-crystallin fractions. As mentioned earlier the two bands of fastest electrophoretic mobility resolved into two components when re-electrophoresed, (as shown in figure 14), for descriptive purposes they have been termed fractions A, B, C and D in order of increasing electrophoretic mobility.

Fraction A was strongly enriched in the three major components designated as 4, 5, 6, found in the delta-crystallin fraction. The two major components found in the alpha-crystallin preparation, designated components 1 and 2, were however present in a much reduced quantity compared to that of the main delta-crystallin aggregate. This fact, taken together with the preponderance of component 4, may explain why its electrophoretic mobility was greater than that of the main delta-crystallin aggregate. The slightly faster fraction B, contains more of component 2, but is more enhanced than A in component 4. Again the three major bands are components 4, 5 and 6.

Fraction C showed a remarkable enhancement of components 4 and 5 together with two strongly staining components, of low iso-electric point. The presence of these components and a decrease in component 6, of high iso-electric point, would explain its high electrophoretic mobility at an alkaline pH. The correlation between high electrophoretic mobility and acquisition of components of low iso-electric point was emphasised even more in fraction D. Only band 4 is present to any extent amongst components of high iso-electric point, whilst components of low iso-electric point

stain more strongly in fraction D than in any other fraction described here.

These results were highly encouraging. Re-electrophoresis into polyacrylamide gels with an increased degree of cross-linkage provided fractions clearly enriched in certain bands, whilst other components were present only in trace amounts. Components designated 1 and 2 were again found greatly enriched in both alpha-crystallin preparations as previously found. Components 4,5 and 6 appeared to be concentrated in delta-crystallin fractions, two of these components 4 and 5 had been substantially enriched in impure fractions of delta-crystallin obtained by gel filtration. Slices obtained from bands of increasing mobility were increasingly depleted in the (alpha-crystallin) components 1 and 2. Moreover slight increases in electrophoretic mobility could be correlated with the increase of components of low iso-electric point or a corresponding decrease in quantity of components of high iso-electirc point. This correlation of molecular charge with electrophoretic mobility would not necessarily be seen where the aggregates differed sufficiently in size so that the separation of protein samples in polyacrylamide electrophoresis was largely on the basis of molecular weight. However separation clearly determined by the molecular weight of the aggregates could only be shown if the iso-electric points of the slower aggregate were lower than that of the band of faster electrophoretic mobility, that is molecular charge effects played a secondary role in the separation procedure. No assessment of the relative effects of molecular charge and molecular size in the fractionation of protein samples in gels with an increased degree of cross-linkage is possible from these results, since the samples also show a tendency towards increasing molecular charge. Fraction D for example contains both weaker staining and fewer components than fraction C

Table 6.

(See figure 15)

IEF gel No.	Sample	Initial Separation technique	Second method of separation	Third method of separation
2	α-Crystallin	repeated gel filtration	agarose electrophoresis	PAGE(2.6% bis)
3	α-crystallin	repeated gel filtration	PAGE (2.6% bis)	-
4	α-crystallin	agarose electrophoresis	PAGE (2.6% bis)	PAGE(3.5% bis)
8 and 9(mixtures)	leading δ-crystallin	agarose electrophoresis	PAGE (2.6% bis)	-
6	intermediate δ-crystallin(1)	PAGE (2.6% bis)	PAGE (3.5% bis)	-
9	intermediate δ-crystallin(2)	PAGE (2.6% bis)	PAGE (3.5% bis)	-

Table 6 shows the respective separation procedures employed on the above samples prior to analysis on iso-electric focusing gels containing concentrated urea. The initials PAGE represent the term Poly Acrylamide Gel Electrophoresis. The percentage of bis-acrylamide monomer is also shown. The electrofocusing results are shown in figure 15.

and consequently would be less retarded by the sieving effect of the polyacrylamide gel, but it would also be expected on the basis of the generally low iso-electric points of its components to be more highly charged than fraction C in alkaline conditions of electrophoresis. Thus the manner in which increasing cross-linkage effects electrophoretic behaviour, by first affecting pore size and consequently migration, or in some other yet undetermined way, remained unresolved by these results.

Further purification and subunit analysis of chick crystallin fractions

The discovery that re-electrophoresis into gels containing 3.5% cross-linkage resolved delta-crystallin aggregates into very sharp bands, composed of comparatively few subunits, as revealed by the iso-electric focusing technique, was utilised to purify the delta-crystallin region that could be isolated from agarose electrophoresis. This subjected a sample separated mainly on the basis of molecular charge to the sieving action of polyacrylamide electrophoresis, once in gels of 2.6% cross-linkage, then finally in gels of 3.5% cross linkage. It was hoped in this way to remove contaminants of differing molecular weight. The effectiveness of the initial agarose electrophoresis could then be directly determined relative to polyacrylamide re-electrophoresis alone.

The conditions for agarose electrophoresis and the identification of delta-crystallin were as described in the Materials and Methods section. Table 6 is a compilation of the various separation procedures used to obtain purified crystallin fractions prior to subunit analysis. Where required the proteins were precipitated *in situ* with 80% saturated ammonium sulphate and subsequently excised. The ammonium sulphate was removed in the usual fashion by dialysis overnight against Tris-glycine buffer. The results of the subunit analyses are shown in figure 15.

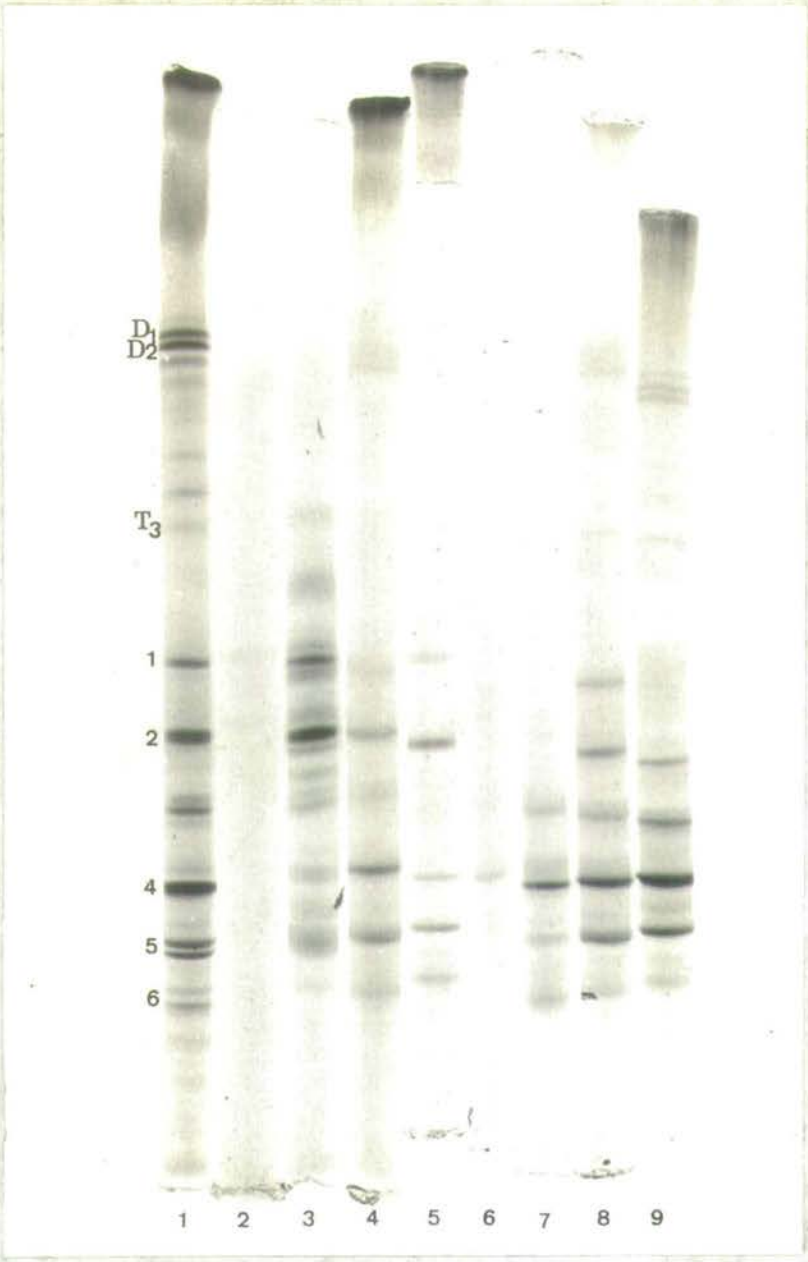


Figure 15. Urea gel electrofocusing of alpha and delta crystallin samples prepared initially by agarose electrophoresis and purified by electrophoresis on polyacrylamide gel (bis/acrylamide ratio 37:1). Samples: 1) Total chick crystallin. 2) alpha (from leading edge in agarose electrophoresis). 3) alpha prepared by polyacrylamide electrophoresis alone. 4) alpha (leading edge) plus delta (leading agarose fraction). 5) major delta fraction. 6) intermediate delta-1. 7) intermediate delta-2. 8) delta (leading agarose fraction) plus int. delta-1. 9) delta (leading agarose fraction) plus int. delta-2. See table 6 for the respective separation procedure employed to obtain each of the samples.

The alpha-crystallin fraction obtained by gel filtration and purified by agarose and polyacrylamide electrophoresis showed only two bands, the components 1 and 2 previously found in all alpha-crystallin preparations, (see gel 2 figures15 and 16). These bands were present in greatly enriched quantity in the alpha-crystallin fraction purified by polyacrylamide electrophoresis only (gel 3 figure 15). Thirteen minor components were also present, mainly of intermediate iso-electric point, but it was clear that the polyacrylamide electrophoresis had removed or reduced components of both high and low iso-electric point.

The delta-crystallin fraction separated by agarose and double polyacrylamide electrophoresis, appeared to be considerably purified relative to the sample of total adult crystallin (compare gel 1 with 5, figure 15), but appeared similar to the major delta-crystallin fraction obtained by polyacrylamide re-electrophoresis alone (gel 4 figure 14). Components 4, 5 and 6 were clearly visable, together with components 1 and 2, associated with alpha-crystallin. Components D_1 and D_2 were only present in very minor quantity. Only two other minor bands between components 2 and 4 could be detected. The subunit analysis of a mixture of the highly purified alpha-crystallin (shown in gel 2, figure 15) and this delta-crystallin revealed no additional bands, only a very slight enrichment of components 1 and 2, emphasising the high degree of purity obtained by the combination of different separation methods (see gel 4, figure 15).

Both intermediate delta-crystallin fractions were prepared from total adult crystallin by re-electrophoresis of aggregates isolated initially in 7.5% polyacrylamide gels containing 2.6% bis acrylamide. Unfortunately the slowest intermediate delta-crystallin fraction showed only three faintly staining bands in the ampholine gel, components 4, 5 and 6 (gel 6 figure 15). These components together with band three were much more

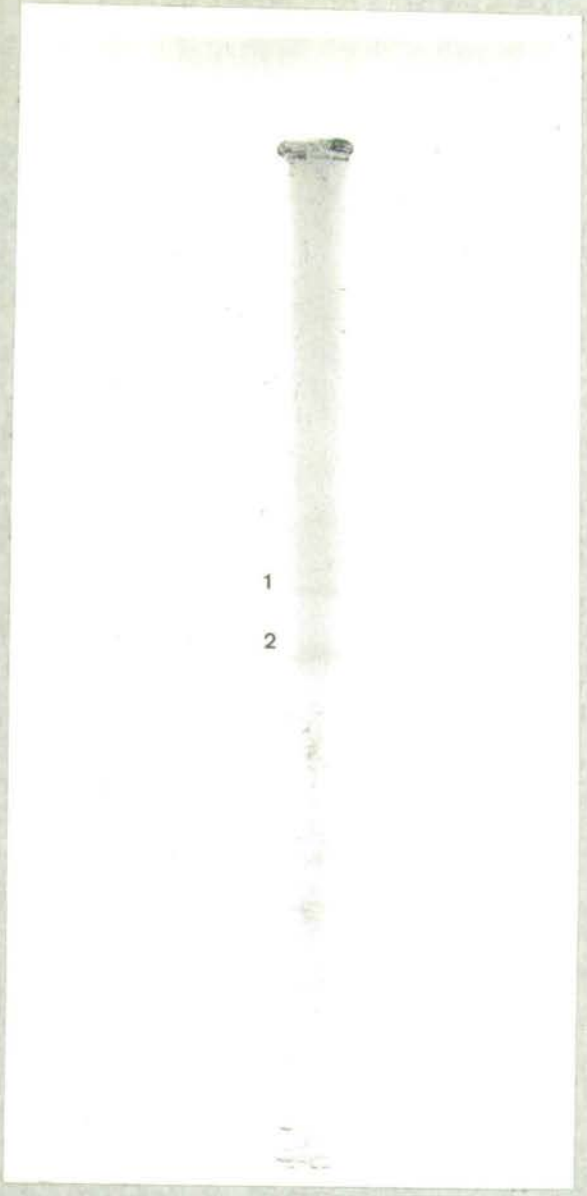


Figure 16. Urea gel electrofocusing of alpha-crystallin taken from the fastest fraction in agarose electrophoresis then further purified by polyacrylamide gel electrophoresis. This fraction (also shown in gel 2, figure 15) shows, albeit faintly, only two subunits, components 1 and 2.

obvious in the faster intermediate delta-crystallin fraction (gel 7, figure 15). Components 1 and 2, associated with alpha-crystallin, were not present in contrast to the main crystallin fraction prepared initially by agarose electrophoresis (gel 5 figure 15) or by double polyacrylamide electrophoresis (gel 4 figure 14). The persistent association of the main delta-crystallin fraction with alpha-crystallin of higher molecular weight would, of course, explain its slower electrophoretic mobility in polyacrylamide gels in non-dissociating conditions, compared to the intermediate delta-fractions. If the alpha-crystallin components 1 and 2 are disregarded then there is an excellent correlation between the main delta-crystallin component, identified from the original schemata devised by R. M. Clayton in this laboratory. As well as emphasising the degree of purity that can be achieved, at least for minor aggregates, by re-electrophoresis in polyacrlylamide gels containing an increased amount of cross-linkage, it also emphasises the validity of the designations of delta-crystallin made in the original work (Clayton, 1969).

The leading edge in agarose electrophoresis of the delta-crystallin region was also doubly purified on polyacrylamide gels, cut out from the final gel of increased cross-linkage, and analysed on ampholine gels together with the two intermediate delta-crystallin fractions. The iso-electric spectrum of these mixtures are shown in gels 8 and 9 (figure 15). The components D_1 and D_2 appeared to be present in slightly greater amounts in the leading fraction compared to the centre fraction cut out from under the delta-crystallin immunological arc. Since any increase of these components of low iso-electric point would increase the charge carried by the aggregates, in electrophoresis of alkaline pH, it is understandable that they were more strongly represented in the faster aggregates of the delta-crystallin region. These components D_1 and D_2 still appeared to be in minor quantities

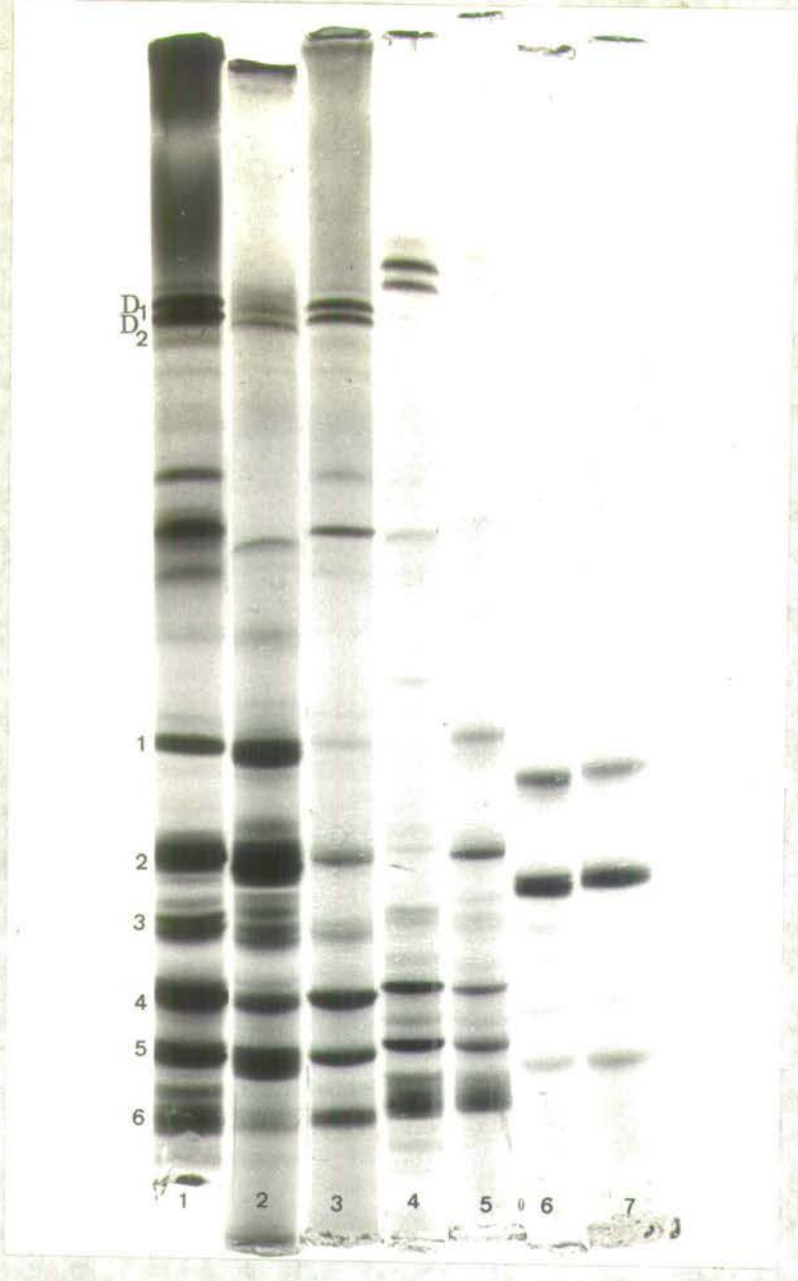


Figure 17. Urea gel electrofocusing of partially purified samples of alpha and delta crystallin fractions. The respective separation procedures used to obtain these fractions are shown in Table 7. Samples:

1) Total chick crystallin.
2) alpha (unpurified from column).
3) delta (unpurified from column).
4) delta (agar and PAGE).
5) alpha (agarose and PAGE) plus delta.
6) alpha - 27 (agarose then PAGE - bis/acrylamide ratio 27:1).
7) alpha - 37 (agarose then PAGE - bis/acrylamide ratio 37:1).

compared to the strongly staining components 4 and 5 shown in gels 8 and 9 (figure 15). Components 1 and 2 were clearly still present in the leading delta-fraction, together with a minor component T_3.

Additional subunit analysis of highly purified alpha and delta-crystallin fractions.

Attention was concentrated on resolving the subunit composition of alpha-crystallin and delta-crystallin since partially purified fractions of these proteins were obtained most readily by gel filtration. Furthermore their behaviour and appearance after *in situ* precipitation in the gel medium by ammonium sulphate was distinctive, and unambiguous. However this distinction could not be made between protein aggregates of high electrophoretic mobility, where fast beta aggregates were to be expected, (Truman et al. 1971a). Although some patterns of enrichment were apparent in the earliest gel electrofocusing results on anodal fractions, the degree of overlap of components between adjacent fractions precluded any attempts to assign these bands of low iso-electric point (and high charge) to any crystallin class. The tentative assignment of certain subunits to particular crystallin classes, outlined in the previous section, was checked by comparing delta-crystallin isolated by agar electrophoresis and purified by polyacrylamide electrophoresis with that previously described, which was initially isolated by agarose electrophoresis. Samples of alpha-crystallin were also prepared in the same manner, in order to compare the effects of different separation procedures in reducing contamination. These final separation procedures are outlined in table 7 and the results of the subunit analysis are shown in figure 17.

The sample of alpha-crystallin obtained by a single passage through the gel filtration system (Truman, 1968) was greatly enriched again in components 1 and 2, although not as pure as a similar sample analysed

Table 7

IEF Gel Number in Figure 17	Sample	Initial separation technique	Second method of separation
2	α - crystallin	single gel filtration	
6	α - crystallin	agarose electrophoresis	PAGE (2.6% bis)
7	α - crystallin	agarose electrophoresis	PAGE (3.5% bis)
3	δ - crystallin	repeated gel filtration	
4	δ - crystallin	agar electrophoresis	PAGE (2.6% bis)

Table 7 shows the respective separation procedures employed on the above samples prior to urea gel electrofocusing. Other details as in Table 6. The electrofocusing results are shown in fig.17.

earlier, (compare gel 2 figure 17 with gel 2 figure 14). However components 6 and D_1 and D_2 appeared depleted when compared with a standard of total lens cystallin. The alpha-crystallin samples obtained from agarose electrophoresis and purified on either polyacrylamide gels of 2.6% or 3.5% cross-linkage appeared very similar. Apart from the strongly staining components 1 and 2, a faintly staining component 5 was also present (see gels 6 and 7, figure 17). Components 3 and 4 were present in very minor quantities. Components 3, 4 and 5 were not observed in the analysis described in the previous section, where a sample of alpha-crystallin obtained by repeated gel filtration was purified by the same separation methods (see gel 2, figure 15).

The delta-crystallin isolated initially by agar electrophoresis and purified on polyacrylamide, had strongly staining components 4, 5, 6 and D_1 and D_2 (gel 4, figure 17). It contained only minor traces of components 1 and 2 when compared to a similar sample obtained by agarose electrophoresis (gel 5, figure 15), although additional components of high iso-electric point were also present. Overall the iso-electric spectrum of this sample was very similar to that of the delta-crystallin obtained initially from repeated gel filtration (gel 3, figure 17), except that it had been purified considerably from components 1 and 2, now firmly identified as components of alpha-crystallin. The sample again had predominant components 4, 5 and 6 and D_1 and D_2 , with five minor components, and one other component between bands 5 and 6.

When the most purified alpha-crystallin fraction (shown in gel 6, figure 17), was co-electrophoresed with a small amount of agar purified δ -crystallin (gel 4, figure 17), the mixture showed the two major components 1 and 2, associated with alpha-crystallin, together with the rest of the bands found in the delta-crystallin sample (see gel 5, figure 17).

Because of the reduced amount of the delta-crystallin sample applied,

some of the minor bands cannot be easily seen in the photograph. But the appearance of the two strongly staining major components 1 and 2, in the mixture, although barely present in the delta-crystallin fraction, emphasised again the reliability and reproducibility of the gel iso-electric focusing technique.

Gel electrofocusing in dissociating conditions of an immunologically pure sample of delta-crystallin.

After the attempts, detailed in earlier sections, to obtain compratively pure samples of alpha and delta-crystallins by combinations of electrophoretic techniques, a sample of delta-crystallin was prepared by repeated gel filtration of chick lens proteins. This particular sample prepared according to the methods used by Truman (1968), appeared when tested by immuno-electrophoresis in agar against an antiserum to the total soluble proteins of chick lens, to be extremely pure (figure 18). The subunit analysis by gel electrofocusing is shown in figure 19, together with various concentrations of a protein sample derived from the nucleus of adult chicken lens. Approximately 50 µg of the delta-crystallin fraction was applied to the gel and seven components were clearly visable: D_1, D_2, 1, 2, 4, 5 and 6 (gel 3, figure 19). If components 1 and 2 are disregarded as alpha-crystallin contaminants, this result would suggest that chick delta-crystallin was composed of five major subunits: D_1, D_2, 4, 5 and 6 in the schemata employed in this investigation. The components D_1 and D_2 were quite strongly ~~proteins from~~ represented in this preparation, since a comparable sample (62.5 µg) of chick lens nucleus (gel 4, figure 19) did not show these components, although they were clearly visible at higher concentrations (compare gels 1 and 2 with 4, figure 19).

Components 4,5, and 6 have been found common to all partially purified samples of delta-crystallin analysed, but the subunits designated D_1 and D_2 were only very minor components in both the main delta-crystallin

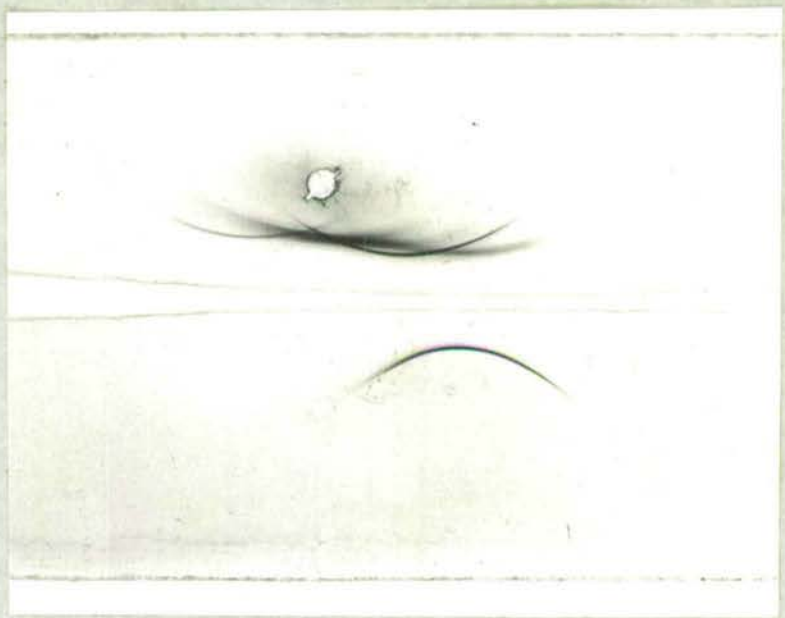


Figure 18. Immunoelectrophoretic pattern obtained with total chick crystallins (upper well) and a preparation of delta-crystallin (lower well). The antiserum was against total chick crystallins and the anode was to the left. With the delta-crystallin sample, the antiserum produced a single precipitin band corresponding to the delta-crystallin arc of the adult chick sample. Approximate loadings: 2.5µl total chick crystallins at 15mg/ml, 2.5µl delta-crystallin sample at 9mg/ml.

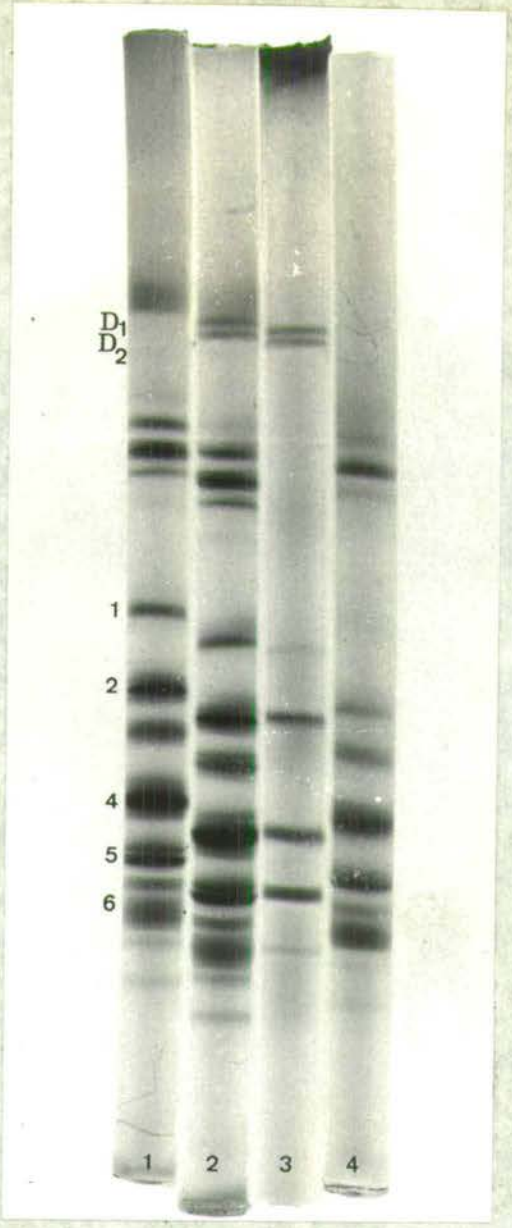


Figure 19. Urea gel electrofocusing of an immunologically pure sample of chick delta crystallin, prepared by gel filtration.

Samples :

1) Chick crystallin from adult nucleus (250 µg).
2) Adult nucleus crystallin (125 µg).
3) delta crystallin (50 µg).
4) Adult nucleus crystallin (62.5 µg).

fraction obtained by double polyacrylamide electrophoresis (gel 4, figure 14) and the fraction obtained from agarose and purified by re-electrophoresis (gel 5, figure 15). They can however be found in considerable amounts in the faster intermediate delta-fractions prepared by polyacrylamide re-electrophoresis (gels 7 and 8, figure 14) and delta-fractions obtained from agar electrophoresis and subsequently purified by re-electrophoresis (gel 4, figure 17).

The results presented in this section, where a delta-crystallin sample, apparently pure by immunological criteria, clearly contained the components D_1 and D_2, argues that they should be considered as subunits of the delta-crystallin protein. However one could also argue that they are components of another class (the beta-crystallins, since they are not found associated with alpha-crystallin components 1 and 2) which remain persistently associated with the major delta-components 4, 5 and 6 and are only removed by severe sieving conditions in polyacrylamide electrophoresis. The classification of these components is reviewed later in the light of results from two separate experimental procedures.

Chapter 6

IMMUNOLOGICAL ANALYSIS AND SUBUNIT COMPOSITION STUDIES OF DIFFERENT PORTIONS OF THE AGAROSE ELECTROPHORETIC SPECTRUM OF CHICK CRYSTALLINS.

Since crystallins have no enzymatic properties, probably the most reliable method of classifying them is by virtue of their antigenic specificity. Consequently throughout this investigation, where possible, samples were classified immunologically prior to any physico-chemical analysis. Numerous subunit analyses of the protein aggregates that could be separated by polyacrylamide gel electrophoresis of chick lens samples, indicated that many aggregates had multiple subunits in common. Even where separations were carried out in dissociating conditions subsequent iso-electric focusing revealed several components in each isolated polyacrylamide slice. Thus no definite or totally specific immunological classification could be given to any aggregates isolated by polyacrylamide electrophoresis of total lens crystallins. All samples of partially purified fractions were of necessity, identified immunologically before subsequent polyacrylamide electrophoresis. Whilst this proved feasible for alpha and delta-crystallins the preparation of reasonably pure samples of beta-crystallin proved difficult. Although chick beta-crystallins have been separated by gel filtration (Truman, 1968) they form a broad peak on many gel filtration media and the difficulty is to separate the leading edge of this from the most retarded portion of the delta-crystallin band. More importantly the beta-crystallins seem to be particularly susceptible to denaturation during gel filtration, and cannot be stored for any great length of time after the initial separation. No reliable beta-crystallin preparations of reasonable purity, obtained by gel filtration techniques, have been obtained for analysis on electro-focusing gels. Furthermore in micro-immuno-electrophoresis the cathodal and anodal beta-crystallins extend mainly over the alpha and delta precipitin arcs, so that good yields of partially purified beta-crystallin could not be obtained by this technique.

However with the classification of several of the subunits apparently complete, it seemed feasible to try and identify beta-crystallin subunits, defined as the polypeptides isolated by iso-electric focusing techniques in dissociating conditions from the protein aggregates found under the beta-crystallin or "long line" precipitin arc. Obviously this classification would be greatly simplified if the immuno-electropherogram could be considerably extended. Thus it was attempted to devise a method of performing extended immuno-electrophoresis for two distinct purposes:-

(a) to identify immunologically the beta-crystallin subunits and

(b) to relate the immuno-electrophoretic pattern of the undissociated lens proteins to the subunit structure of the crystallins.

A macro-scale technique for immuno-electrophoresis of chick lens proteins.

In order to obtain sufficient material for subsequent subunit analysis horizontally level, defatted glass plates, 18 cms wide by 30 cms long and 0.4 cms thick, were covered in initial experiments with a 3 mm thick layer of 1% ($^{W}/_{v}$) agarose. The agarose was dissolved in high resolution buffer (HRB) (Aryonsson and Gronwall, 1957) exactly as described for micro-scale immuno-electrophoresis in the Material and Methods section. After gel formation, sample wells, o.4 cms in diameter, were cut by means of a Pasteur Pipette rim top at a distance of 10 cms from the cathodic edge of the glass plate. Approximately 25 wells at a distance of 1 cm from each other were employed in each run. 400 µg of protein were dispensed by means of a Hamilton syringe into each sample well. The preparation of plates and pattern for macro-immuno-electrophoresis was thus similar to that described in Grabar and Williams (1953), who employed agar plates and electrophoresis times of about 4-6 hours. Agarose was employed here, since theoretically at least, it does not contain ionisable groups which cause electro-osmotic flow within gels. However at room temperature

with a voltage of 5v/cm, with pH 8.9 agar, considerable electro-osmotic flow occured. This led to a considerable thinning of the agarose layer in the centre of the plate within a few hours. Consequently the migration of the proteins was completely halted, and the agarose layer was so thin and dry that antibody troughs could be cut only with difficulty. In many cases the agarose layer was too thin and devoid of buffer to permit satisfactory diffusion of the antibody molecules into the gel. No significant improvement was observed if the electrophoretic run was performed at 4°C. Under these conditions the run could be continued only for about 5 hours. Figure 20 shows the immuno-electropherogram of chick crystallins electrophoresed for 5 hours at 4°C. under the described conditions. Whilst the immuno-electropherogram has not been greatly extended in length, it should be noted that identical samples electrophoresed together on the same plate behaved very similarly. No obvious local differences in the immuno-electrophoretic patterns were detected other than at the very edge of the glass plates. Subsequently sample wells were cut at a minimum of 3 cms. from the top and bottom edges of the glass plate.

Whilst the immunological data indicated that comparable electrophoretic conditions obtained over all but the very edges of the agarose plate, the extent of the electro-osmotic flow in the agarose gel seemed to preclude the possibility of extending sufficiently the period of electrophoresis. Quast (1971) examined twelve commercial preparations of agarose for electro-osmosis and all showed electro-osmotic flow. He ascribed the presence of charged groups responsible for the electro-osmosis to contaminants of ionisable macro-molecules such as agaropectin.

The author noted that electro-osmotic flow in certain commercial batches may be reduced by treatment with anion-exchange resin.

Figure 20. Stained electropherogram of total chick crystallins fractionated by electrophoresis in 1% agarose for 5 hours at 4°C, under the conditions described in the text. After the run was terminated because of electro-osmotic effects, a test sample was stained with 1% Amido Black, as described in the Materials and Methods section.

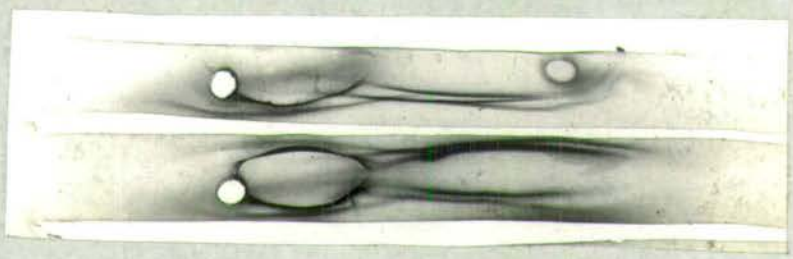


Figure 21. Extended immunoelectrophoretic pattern obtained with a thicker (9mm) agarose plate. Electrophoresis in 1% agarose was continued for 16 hours at 4°C, using the High Resolution Buffer of Arrønsson and Grønwall, (1957). The troughs contained antiserum to total chick crystallins (JHI). The beta-crystallin line is considerably lengthened in these conditions (compare the immunoelectropherogram shown in fig. 12a).

Some authors have enhanced separation and obtained faster movement of fractions by using higher field strengths, made possible through the use of cooling devices. Wieme (1965) has carried out electrophoresis below light petroleum ether whose evaporation and consequent cooling effect enabled a constant temperature to be maintained. However neither the requisite apparatus nor the petroleum ether were available at the time of the investigation.

Resort was subsequently made to a simpler stratagem to enable electrophoresis to be maintained for a period of about 16 hours. If the glass plates were covered with a 9 mm thick agarose layer, although electroendomosis was still severe, the agarose remained sufficiently thick to allow continued migration of the protein fractions, whilst antibody troughs could be cut with ease. Perfectly adequate immuno-diffusion can take place in such conditions and a considerably extended immuno-electropherogram can be obtained as shown in figure 21. Again the immuno-electrophoretic patterns indicated that the samples of total chick lens proteins appeared to electrophorese in highly similar fashion regardless of position on the large glass plate. A high degree of reproducibility between samples is necessary if comparable regions from different sample tracks on theplate are to be amassed together to provide sufficient material for further biochemical investigation. Considerably lengthened and enhanced resolution of the beta-crystallins can be obtained using this simple modification. Figure 21 represents however the maximum resolution obtained with the particular buffer employed (High resolution buffer - Arronsson and Gronwall, 1957).

The ionic strength of the buffer is also of great importance in agarose gel electrophoresis, too high a buffer concentration may lead to heating effects, whilst with too low a concentration, variation in pH

may occur. The following buffer (pH 8.6) gave excellent results, in reducing the degree of endosomosis: sodium barbitone, 10.31g/litre, barbituric acid, 1.84g/litre, sodium acetate, 4.1g/litre, methiolate, 0.1g/litre, diluted 1 : 4 before use. The immunoelectropherogram, as shown in figure 22, can now be extended over a distance of 10cms., whilst inter-sample variation on the same glass plate appeared minimal. Employing this buffer, the final conditions chosen for an overnight run were a total voltage across the plate of 100 volts, continued for 2 hours, followed by 50 volts for a further 15 hours. These conditions ensure that the fastest migrating fraction reaches about 2 cms. from the anodal edge of the plate.

With an agarose gel of this original thickness (9mm) it is important to employ extensive and repeated washing of the gel in 0.9% ($^{W}/_{V}$) physiological saline to ensure that all unreacted antigen and antibody are removed from the gel. This was normally performed over a period of 24 to 48 hours, with several changes of saline.

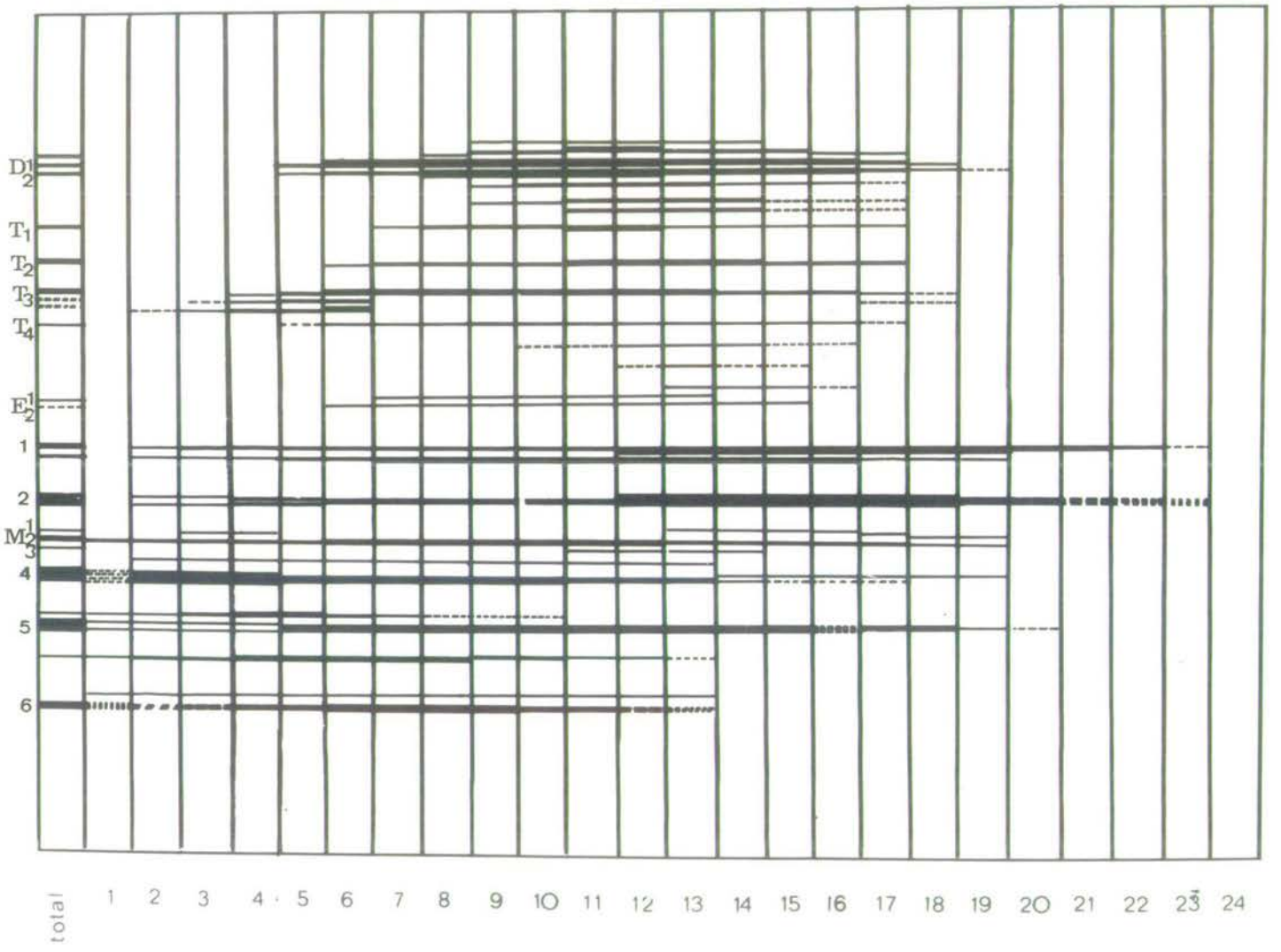
The treatment of agarose strips prior to iso-electric focusing was as described fully in the Materials and Methods section. The gels containing the protein re-run from various regions of the first dimensional separation in agarose were then stained with the quantitative Fast Green dye. The iso-electric spectrum of each agarose fraction was then recorded, thus representing the subunits present in that particular agarose region, whilst the immuno-diffusion analysis of control samples, indicated the immunological relationships of the undissociated proteins in any particular agarose region.

The development of a suitable macro-scale technique for extended agarose electrophoresis, together with a highly resolving technique of iso-electric focusing in dissociating conditions thus made possible an attempt to correlate directly the protein subunits involved in a particular

assemblage that could be identified immunologically. Since the various fractions could be analysed simultaneously it meant that the numbers and type of subunits associated with any particular precipitin arc could be compared within a single agarose electrophoresis. The diagram shown in figure 22b represents the subunit analysis of polymers whose assemblage can be classified on the basis of the immuno-electropherogram shown above (figure 22).

Several factors must be borne in mind when interpreting this figure. The immuno-electropherogram represents the direct immuno-electrophoresis of chick crystallins with a particular antibody 42W, prepared as an anti-total chick crystallin serum. But the relative titres of antibodies to individual constituent antigens vary with the particular anti-total sera used. For example it was routinely found that a comparable anti-total chick crystallin sera, JHI, was more potent against faster delta-crystallins, and the immunological arc of the delta-crystallin was considerably more extensive than that obtained with the serum 42W. The most likely explanation for this finding was that serum JHI was detecting the smaller and faster delta-crystallin aggregates partially separated during the agarose electrophoresis. This point is made to emphasise that antigens found intermediate to two discrete immunological arcs may have determinants in common with either of these regions, but may not be detected since a specific antibody may not have a high titre to the particular assemblages in which the antigen is contained. A more extensive overlapping of immunological arcs may be revealed by a second antibody. Where the titre of an antibody to particular molecules or assemblages of molecules is low, the resultant soluble antigen-antibody may easily be washed out in the subsequent procedures of immuno-electrophoresis. The insoluble precipitin line remaining will be characteristic of a particular antibody. But the antibody is here directed only against the 400 µg of protein electrophoresed in a single well. Since only a small amount of agarose could be applied to an ampholine gel, half centimetre

Fig.22b. Subunit analysis by urea gel electrofocusing of different fractions of total chick crystallins separated by macro-scale agarose electrophoresis. Following agarose electrophoresis of total chick crystallin (see text) 0.5 cm agarose skips were cut out, dried down and dissociated in 8M urea (see Materials and Methods section). Each strip was then electrofocused in dissociating conditions and the ampholine gel stained with Fast Green. The diagram thus represents the iso-electric spectrum of the subunits present in each agarose strip.



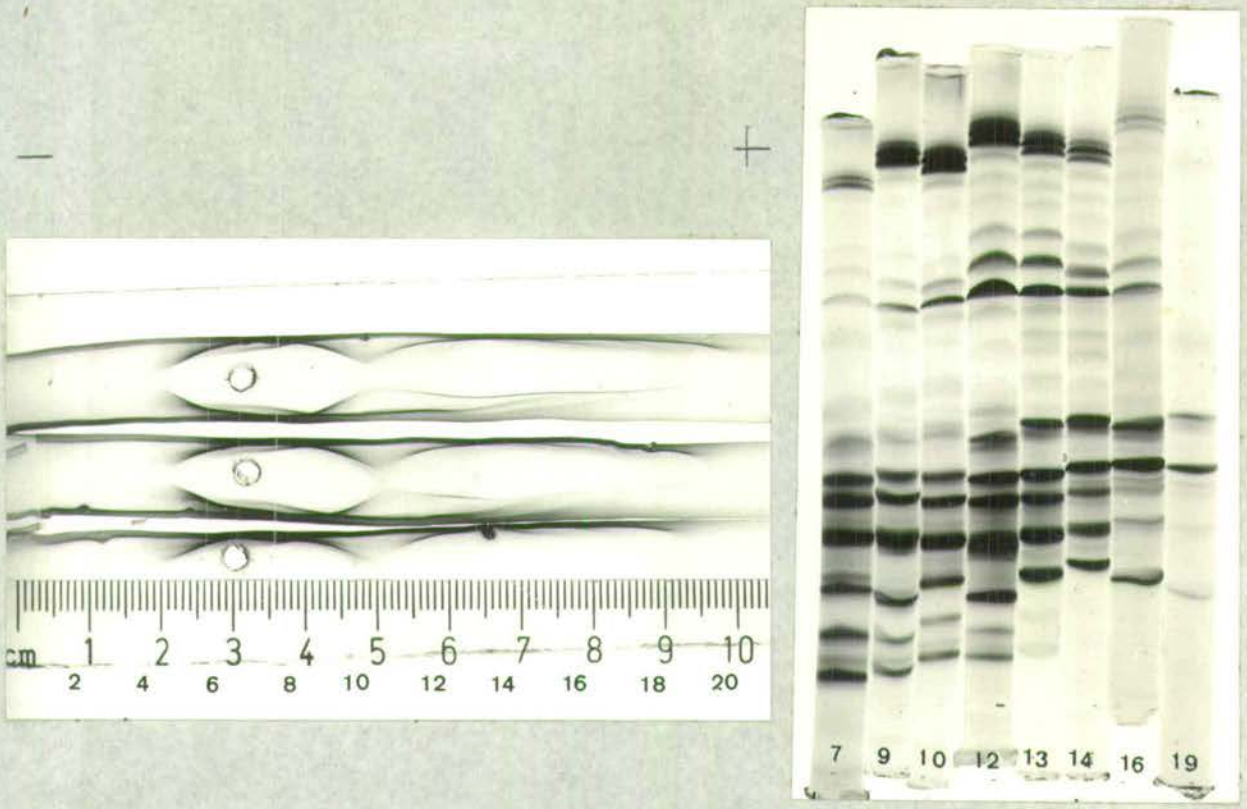


Figure 22a. Immunological analysis and subunit composition studies of the agarose electrophoretic spectrum of chick crystallins. Left: Extended immunoelectrophoretic pattern obtained with a Tris-Veronal electrophoresis buffer. Electrophoresis in 1% agarose was continued for 16 hours at 4°C. Other modified conditions were as described in the text. Test portions of the electropherogram were then cut for immunological testing against antiserum to total chick crystallins (42W).

Right: Subunit analysis of agarose fractions. Following agarose electrophoresis, 5mm strips of agarose were prepared for analysis by urea-gel electrofocusing (see Materials and Methods section). The gels shown represent the isoelectric patterns of specified agarose fractions. The analysis of the entire agarose electropherogram is shown in diagrammatic fashion in fig. 22b.

strips from about 25 separate electrophoretic runs on a single agarose plate, were required to produce sufficient starting material. In a single run the quantity of a particular antigen in a specific electrophoretic region may be insufficient for effective binding of antibody, but when concentrated several times the presence of the antigen will be clearly revealed in gel electrofocusing. Thus a pattern of continuity of particular subunits between adjacent fractions will be more readily detectable through the gel electrofocusing technique, since the immuno-electrophoretic analysis depends on the detection of much smaller amounts of antigenic material complexed in aggregates of various charges and sizes.

Prior, then, to the interpretation of figure 22 two major considerations must be borne in mind:-

(i) the degree to which discrete immunological arcs appear to overlap is characteristic of a particular antibody, and may appear extensive or limited depending on the anti-serum used.

(ii) minor amounts of antigen, complexed in various assemblages may not be detected immunologically. But since the aggregates are concentrated and dissociated prior to gel electrofocusing, particular subunits will be easily detected and the degree of continuity of such antigens in adjacent electrophoretic fractions may be more readily estimated .

The fractions corresponding to the centres of particular immunological arcs should of course show definite enrichments of subunits of specific crystallin classes, and this, in fact, appeared to be the case as outlined below.

ALPHA-CRYSTALLIN - The two components 1 and 2, previously found to be characteristic of all alpha-crystallin preparations, were found to be the most highly charged component of all and in fact can be detected in fractions 2 cms. distant from the leading portion of the alpha-crystallin arc, emphasising the validity of the considerations made above. Fractions

12 to 18 were extremely enriched in these components and these fractions correspond directly to the main portion of the alpha-crystallin precipitin line. These two components were present in minor quantities even in the very slowest fraction behind the well. Several factors, or more probably a combination of these factors, may account for the widespread distribution of these alpha-crystallin subunits. Firstly even in 1% agarose, some separation of aggregates on the basis of molecular weight is bound to occur, creating a tailing or smearing of particular components. This phenomenon is well known from preparations of eletrophoresed proteins stained immediately after electrophoresis. A typical pattern will reveal a major staining region connected to the sample well by an extensive tail. Secondly at the pH of 8.6 employed in the electrophoresis, a certain amount of denaturation will occur and dissociation and re-association phenomena may well produce aggregates of a virtually continuous range of size and charge. Furthermore some components may form minor constituents in aggregates whose electrophoretic behaviour are largely dependent on the physic-chemical properties of the bulk components. Minor components may then be carried along in the subsequent electrophoresis of such aggregates, particularly if there is a specific association of certain subunits with crystallin subunits of a second class. Since the alpha-subunits found behind the well were found in minor quantities compared to those fractions of a more cathodal nature, this explanation of electrophoretic behaviour, through entrapment in aggregates of a cathodal nature, appears reasonable.

DELTA-CRYSTALLIN - The major portion of the delta-crystallin arc extends over fractions 6 to 9. All four of these fractions show components 4, 5 and 6 present in major quantities. However the distribution of these components suggests that a certain degree of separation between these subunits has occured during the electrophoresis. Component 4 is greatly enriched in fractions 2 to 4, very close to the cathode. Fraction 5 in

comparison extends anodally almost as far as the two alpha-crystallin components 1 and 2, and is only found in minor quantities in highly cathodal fractions. Component 6 is enriched in fractions 6 to 9, corresponding directly to the delta-crystallin arc. The quantities of component 6 that extends anodally are comparable to those found in the more cathodal fractions.

COMPONENTS D_1 and D_2 - The distribution of these components is more complex than the delta-crystallin subunits just described. D_1 is strongly represented in fractions 6-12, including the majority of fractions under the delta-crystallin arc. However D_2 is found predominantly in fractions 8 to 12, and both components extend anodally to a considerable degree. These components may represent the faster or smaller aggregates detected with antibody JHI, which detects a more extensive delta-crystallin arc than the antibody used here, 42W. It should be noted that these components D_1 and D_2, as expected of proteins with low iso-electric point, predominate in the faster electrophoretic fractions of delta-crystallin samples obtained by single gel filtration (see gels 7 and 8, figure 14). Their distribution in the immuno-electropherogram and their electrophoretic behaviour could also lead them to be considered as beta-crystallins which remain rather persistently associated with the main delta-crystallin subunits. The main case for including them as delta-crystallin subunits is based on their strong representation in a sample, identified immunologically as delta-crystallin, which contained very few subunits (see gel 3, figure 18). A sample of the total soluble proteins from the chick lens nucleus, at approximately the same concentration, did not reveal these comparatively minor components, which were clearly visible at higher concentrations (compare gel 4 with 2, figure 18), (The classification of these components is reviewed again in chapter 8 in the light of further preliminary results).

Thus whilst these results confirmed the classification of components 4, 5 and 6 as belonging to the delta-crystallin class, they also revealed that particular subunits may have restrictive and distinctive electrophoretic

mobilities in agarose, whilst others may show an extensive range of distribution. The significance of this phenomenum is discussed later. Secondly the distribution of say, components D_2 and D_4 were sufficiently distinct to suggest that a certain separation of aggregate types is occuring in extended selective assemblages found in vivo with characteristic patterns of interaction between certain subunits, that are not totally disrupted in agarose electrophoresis. Alternatively, but not exclusively, some aggregates may be constructed randomly during homogenisation and separation procedures, with no specific forms of interaction between subunits. Such aggregates would be expected, on average, to be less cohesive, and consequently may not persist during extended electrophoresis, but separation will occur mainly on the basis of molecular charge and partly on the basis of molecular weight. Whilst the data shown here can throw no direct light on the specificity or otherwise of interactions between subunits of separate crystallin classes, it is significant that component 5 migrates almost as fast as the alpha-crystallin component 1 and 2. It is just this component that is found even in highly purified alpha-crystallin samples (see gels 6 and 7, figure 17). Only leading regions of alpha-crystallin samples separated by agarose could be purified free of this component (see gel 1, figure 16). Yet since alpha-crystallin has a greater molecular weight and is more highly charged than component 5 this result cannot be explained as solely due to similar physio-chemical properties. This persistent association may reflect a specific interaction between the alpha-crystallin components 1 and 2 with the delta-crystallin subunit, component 5.

BETA-CRYSTALLIN - Much evidence has accrued from this laboratory to suggest that chick beta-crystallins are a family of heteropolymers, showing a range of sizes and electrophoretic mobilities, which are antigenically related to each other, partly by containing subunits in common with other members of the class (Clayton and Truman, 1967, 1974;

Clayton, Campbell and Truman, 1968; Clayton, 1969; Truman and Clayton, 1974). As described in the Introduction, the beta-crystallins appear very polydisperse electrophoretically, and in immuno-electrophoresis form an extensive precipitin arc.

The most intensly staining region of the beta-crystallin arc is found in fractions 10 to 13. Apart from the components already classified as alpha or delta-crystallin subunits, three components T_1, T_2 and T_3 were strongly represented in fractions 11 to 12. These are the components found enriched in the polyacrylamide fractions of highest electrophoretic mobility at alkaline pH. Such fractions appear in considerable quantities when purified preparations of beta-crystallin are electrophoresed (Truman et al. 1971; Truman and Clayton, 1974) although the results reported in earlier sections of this work indicated that delta-crystallin subunits could also be isolated from such anodal aggregates where total crystallin was applied to the gel. A fourth component T_4, although not present in such quantity as T_1, T_2 and T_3, was also routinely found in the fastest (anodal) electrophoretic fractions. It showed a very similar electrophoretic distribution in agarose to the components T_1, T_2 and T_3. Components E_1 and E_2 showed a restricted electrophoretic range suggesting they may be beta-crystallins rather than alpha or delta-crystallins, but this conclusion is extremely tentative. Similar claims might be made for the very minor components T_5 and T_6.

OTHER COMPONENTS - Components M_1, M_2 and M_3 were not definitively assigned to a particular crystallin class prior to this experiment. Component M_2 extended throughout the whole electrophoretic range, but the pattern of enrichment in fractions 6 to 12 was compatible with both the delta and beta immunological criteria. The classification of these components is is reviewed in chapter 8 in the light of further experimental results.

The minor component 1b is enriched in fractions 12 to 16, suggesting it may be an alpha-crystallin component. Although it has an extensive electrophoretic range similar to components 1 and 2, no other evidence has accrued from further experiments, so any assignment remains highly tentative.

This attempt to isolate different portions of the agar electrophoretic spectrum of chick crystallins and subject them to both subunit analysis and immunological analysis helped confirm the classification of a number of components, whilst indicating the likely classification of others. A diagram of final assignment of the various components to their crystallin class is given in chapter 8, after consideration of some other experimental results. The overall results on subunit assignation are considered, together with other authors' findings, in the Discussion section.

Chapter 7

ISO-ELECTRIC FOCUSING OF CHICK CRYSTALLINS IN NON-DISSOCIATING CONDITIONS

Although electrofocusing chick crystallins in the presence of 6 M urea did not present any major problems, the development of a reproducible analytical gel technique for iso-electric focusing in non-dissociating conditions proved more difficult. Initially the undissociated protein sample was applied to the top of the gel in exactly the same manner as described for urea dissociated samples. The sample was then focused overnight at room temperature over the pH range 3-10 according to the gel electrofocusing method of Wrigley (1968). Severe wilting of the gel occured and in some cases the gel actually burnt. The zone of wilting, about one-third of the gel length from the origin appeared to be the region where many of the major components were focusing extremely closely together.

The reason for this effect is not clear but since no salt ions are present in this analytical technique, the very low ionic strength may exaggerate the electro-osmotic flow within the gel, leading to micro-convection at the glass surface (Ui, 1971a). This may induce distortion of bands or if local heating effects are too severe, wilting and burning of the polyacrylamide.

In addition however it was observed after protein staining, that the bulk of the sample had remained at the top of the gel, only a minor proportion of the sample had entered the gel.

The severe heating effects observed at room temperature could be largely overcome by performing the gel electrofocusing at $4^{\circ}C$ but a slight thinning of the gel in the major region of focusing was occasionally observed. However the amount of sample entering the gel was considerably reduced in runs performed at $4^{\circ}C$. One possibility was that the 7.5% polyacrylamide gel was exerting a significant molecular sieving effect,

thereby delaying or preventing the crystallins from reaching their iso-electric point in the pH gradient. However, reducing the total polyacrylamide gel concentration to 3.75% to minimise the degree of restriction by the gel did not significantly increase the amount of protein entering the gel.

Bours (1971) found that chick alpha-crystallin tended to precipitate at its iso-electric point in gel electrofocusing (especially if applied cathodally), and it seemed possible that the samples of total chick len crystallins investigated here were similarly precipitating in conditions of low pH. When the crystallin sample was incorporated directly into the gel mixture prior to polymerisation highly reproducible results could be obtained. Thus this simple alteration in loading procedure, described routinely in the original paper on gel electrofocusing (Wrigley, 1968) when combined with a running temperature of 4°C to combat local overheating effects, made possible the analysis of chick crystallins by iso-electric focusing in non-dissociating conditions. Haglund (1971) pointed out that there are many examples of proteins which are insoluble or become denatured in pure water, yet show considerable solubility in a pH gradient. The aminocarboxylic acids have been shown in some cases to provide a protective stabilising effect on proteins susceptible to precipitation (Vesterberg, 1970; Wadstrom and Mollby, 1971).

Thyroglobulin, for example, is known to be insoluble at its iso-electric region, and the iso-electric point derived from electrophoretic mobility values had to be estimated by extrapolation. In solutions of carrier ampholytes, however, thyroglobulin is completely soluble even at the iso-electric point. This measured increase in the solubility of iso-electric thyroglobulin may be due to both the higher dielectric constant of an ampholyte solution and the absence of salt (Ui, 1971a).

Unfortunately the effectiveness of this simple modification was

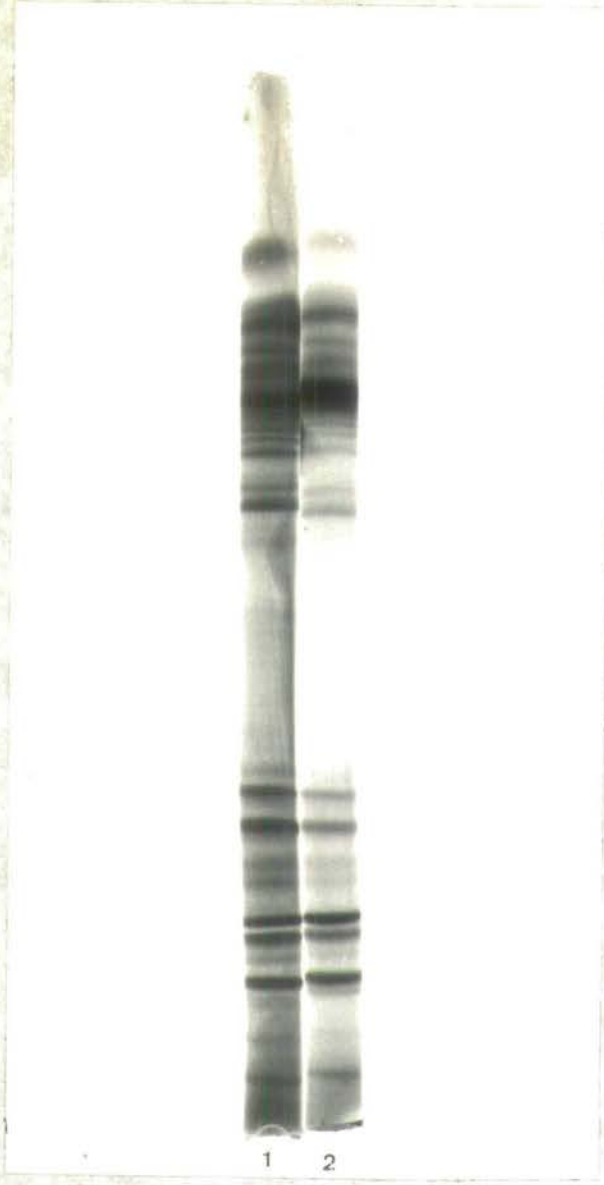


Figure 23. The iso-electric focusing pattern of total (adult) chick lens crystallins in non dissociating conditions. Proteins (at 500 µg per gel) were separated on 7.5% polyacrylamide gel, 1% Ampholine carrier ampholytes, pH range 3.5 - 10. Gel 1 is stained with Coomassie Blue, Gel 2 with Fast Green.

discovered long after the technique of gel electrofocusing in concentrated urea had been completed. Apart from the obvious absence of urea in any of the isolation and preparative procedures, all the minor modifications employed in the method of subunit analysis by gel electrofocusing in urea were utilised including the routine use of an ampholine overlayer, containing 2 - Mercaptoethanol, as both a pre-run solution and protective layer.

Samples were routinely applied by micro-syringe at 500 µg per tube, twice the amount normally used for subunit analysis. The iso-electric spectrum of chick lens proteins obtained in these conditions is illustrated in figure 23.

Using the stain Coomassie Blue, a minimum of 24 protein bands could be detected including a zone of 7-8 tightly compacted bands in pH 4.9-5.3 region. Six major bands and four minor bands were well separated in the pH 6.5-8.0 region. This remarkable number of protein components obtained in non-dissociating conditions emphasises again the remarkable resolving power of the gel electrofocusing technique. The pattern obtained was very similar to that achieved by iso-electric focusing the chick lens crystallins in thin layer 5% polyacrylamide gels (Bours and van Doorenmaalen, 1970; Bours, 1971).

The similarities of the pattern obtained in these flat gels of lower polyacrylamide concentration suggested that a significant retardation of protein through molecular sieving effects had not occured in the 7.5% polyacrylamide rods. In retrospect it was noted that the method of sample application employed by these authors allowed the protein sample to soak into the polyacrylamide plate prior to transfer to the electro-focusing tank, and thus was equivalent to the loading procedure finally employed in these studies. In this manner precipitation of samples prior to iso-electric focusing in gels could be avoided but the alpha-crystallins of the chick lens are known to precipitate in column iso-

electric focusing procedures (Bours et al. 1970).

When samples loaded directly onto the gel rods were compared to those incorporated within the gel, it was noted that proteins ran to identical iso-electric points, again suggesting that any molecular sieving effects occuring within the gel were overcome with time. But since considerably less protein appeared focused in the gel by the former method, it seemed likely that the major restrictive factor in these conditions was the aggregation of the bulk of the proteins at the gel origin. Direct incorporation of the sample into gels of 3.75% polyacrylamide gave an identical iso-electric spectrum to that of samples analysed on 7.5% polyacrylamide gels, emphasising that any molecular sieving effect of the gel did not affect the final iso-electric point of any component. These considerations of sieving effects were necessary since in undissociated samples of chick lens proteins the largest components, the alpha-crystallins, may have molecular weights of over 300,000 daltons. An identical iso-electric spectrum was obtained regardless of the position of the sample incorporated into the gels suggesting that no denaturation was taking place of proteins subjected to a pH far from their iso-electric point. The phenomena of denaturations in such conditions is known to occur with several proteins (Lewin, 1970).

Two other possible sources of artefacts can also be ruled out because of the similarity of these results with those obtained by Bours (1971). Firstly the traces of salt introduced within a sample volume of 8.3 µl and effectively diluted a further 250 times during the course of the gel electrofocusing (in a total gel mixture of 2 mls. per tube), did not appear to affect, the iso-electric results. Lyophilised samples dissolved in pure water showed a similar spectrum (Bours, 1971). Secondly, although artefacts can arise from interactions between the protein sample and the ammonium persulphate used for polymerisation (Brewer, 1967; Fantes and Furminger, 1967), these can be excluded here

since the chemical polymerisation technique used here gave comparable results to those obtained where the gel was photopolymerised with riboflavin as catalyst (Bours, 1971).

Subunit analysis of crystallin components initially isolated by gel electrofocusing in non-dissociating conditions.

Following the establishment of a reproducible technique for gel electrofocusing the chick lens crystallins in non-dissociating conditions, an attempt was made to identify the major components revealed by this method. The major protein bands precipitated by soaking the ampholine gel in 80% saturated ammonium sulphate, were excised and subsequently prepared in the usual manner for subunit analysis in urea-containing electrofocusing gels. A total of 13 single bands and 3 regions containing several closely compacted bands, could be routinely isolated after precipitation *in situ* with ammonium sulphate (see figure 24). The result of the subunit analysis of the single protein bands obtained by gel electrofocusing the crystallins in the absence of urea is shown in figure 25. Similarly the subunit analysis of the polyacrylamide slices containing multiple bands is shown in figure 26. These results are summarised in diagrammatic fashion in figure 27, which thus represents the subunit analysis of each major region obtained by gel elctro-focusing chick crystallins in non-dissociating conditions. As expected the intensity of staining of a particular subunit varied directly with the concentration of the original undissociated protein band in the poly-acrylamide slice, so that no great significance can be placed on the changing patterns of intensities. However there was a clear correlation of the original iso-electric point of a particular band, composed of undissociated protein, with the iso-electric points of its subunit components. As shown clearly in figure 25 the protein components of highest iso-electric point in non-dissociating conditions (bands 11-16) are composed almost entirely of subunits of very high iso-electric point.

Figure 24. Diagram of the electrofocusing pattern, in non-dissociating conditions, of total chick crystallins, after precipitation of protein components in 80% saturated ammonium sulphate.

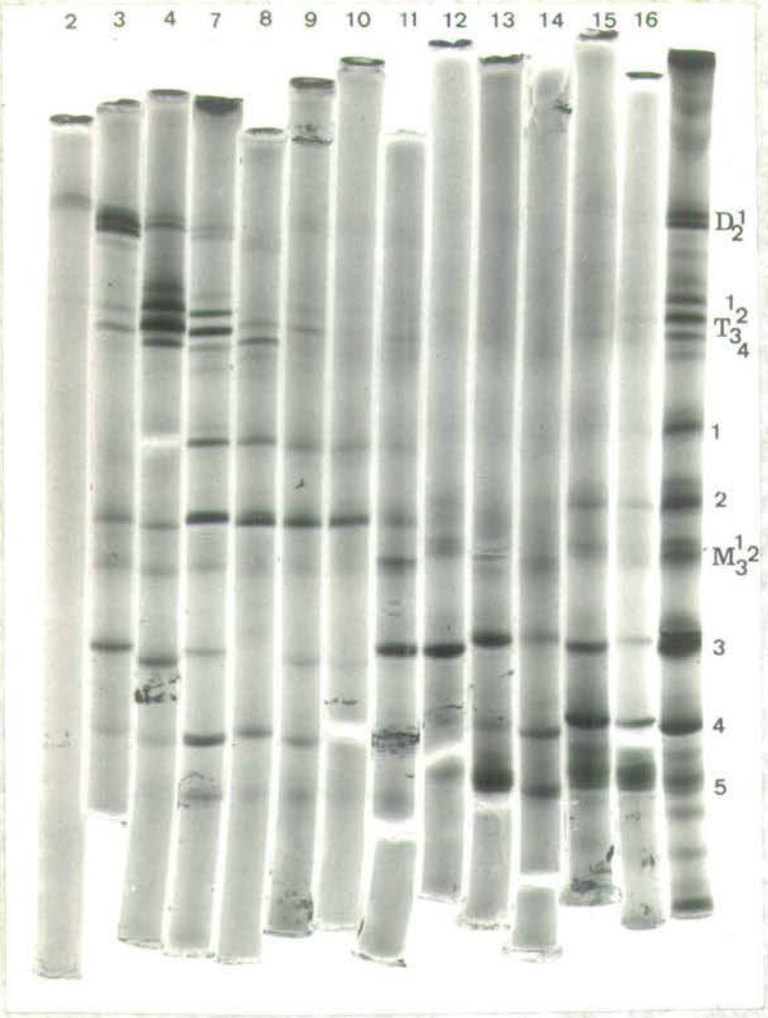


Figure 25. Iso-electric focusing in the presence of 6 M urea of the separate protein bands of total chick lens crystallins isolated initially by gel electrofocusing in the absence of urea, Ampholine range pH 3.5 - 10 (see text). The numbers above the gels represent the band designations shown in figure 24. For comparison is shown a sample of total chick lens crystallins (250 µg), dissociated in 8 M urea prior to iso-electric focusing.

The undissociated protein components of lowest iso-electric point (bands 2-8) contain subunits of low iso-electric point, not found in any quantity in complexes of higher iso-electric point. The increasing predominance of subunits of high pI as the iso-electric points of the undissociated polymers increase would not necessarily be seen if polyacrylamide gel was producing any significant molecular sieving effect, that is if components were being separated partly on the basis of their molecular size.

More importantly, gel electrofocusing of crystallins in the absence of urea is shown by subunit analysis to separate complexes containing different classes of crystallin, rather than homogeneous complexes composed of a single type of crystallin. For example fraction 4 contains multiple bands (figure 25), many of the major subunits found in the sample of total adult crystallin, component 3 is represented in fractions 3 to 16, as are bands M_1, M_2 and M_3. Other components do however show a more restricted distribution. Components 1 and 2, identified previously as alpha-crystallin are distributed mainly in fractions 3 to 10 covering the pH range 4.5-5.9 . Components T_1, T_2, T_3 and T_4 are found mainly in fractions 3 to 8, in the pH range 4.5-5.5 . Whilst components 3, 4 and 5, identified as delta-crystallin are more widely distributed, they represent the major polypeptides found in fractions 11 to 16, covering the pH range 6.5-8.1 . Thus the overall distribution of the subunits indicated clearly that many of the discrete components isolated by gel electrofocusing in non-dissociating conditions were heteropolymers, possibly arising by protein aggregation in the course of homogenisation. Alternatively such heteropolymers may exist naturally within the lens.

If such assemblages were built up randomly, one might expect the complexes to exhibit a variety of iso-electric points dependent on the proportions and nature of the subunits composing the polymer. However the reproducibility of the pattern of crystallins electrofocused in non-dissociating conditions suggests that certain assemblages may be

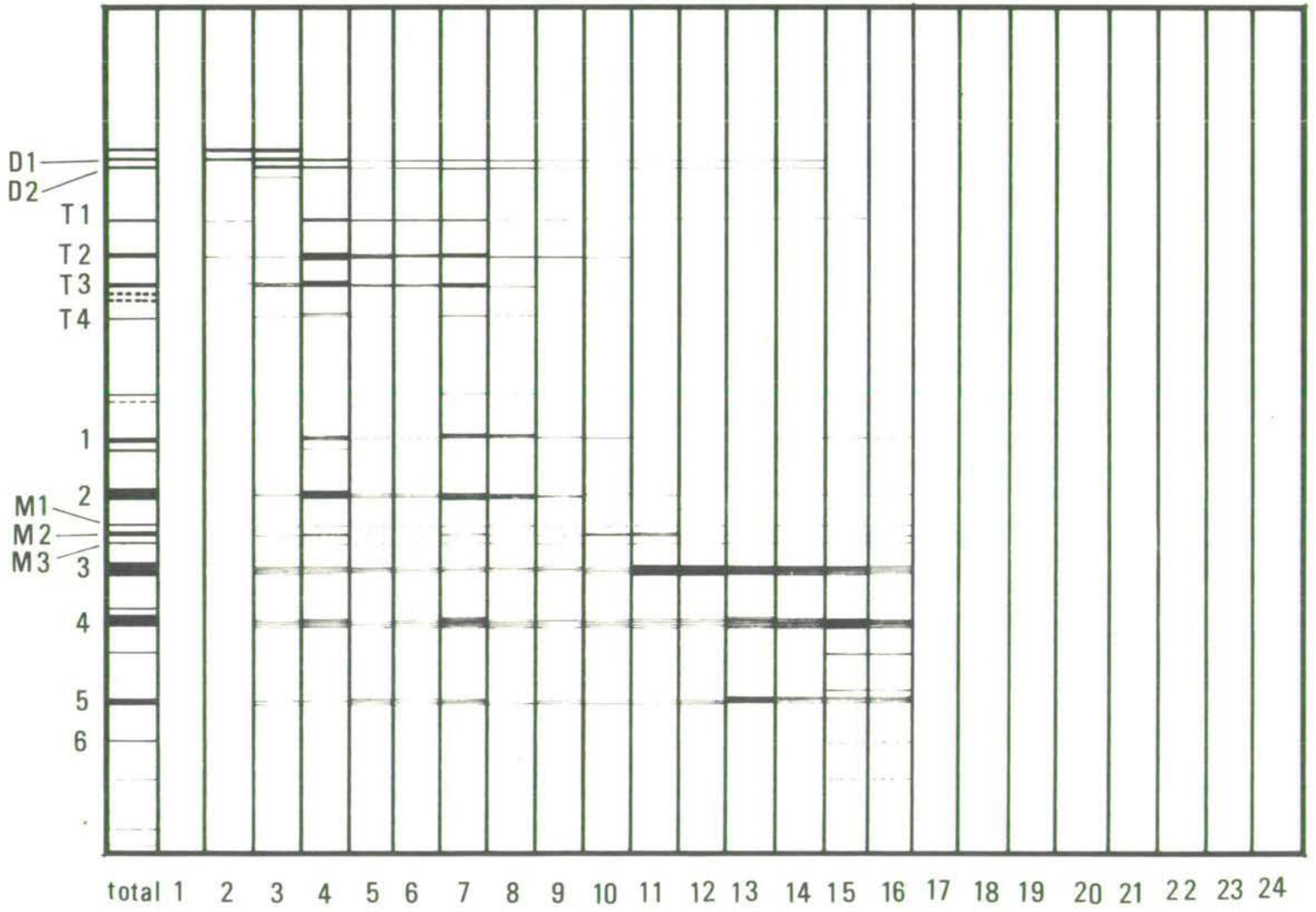


Figure 27. Diagrammatic representation of the isoelectric focusing patterns obtained in the presence of 6M urea of the major regions (nos.1-16 of fig.24) isolated initially by electrofocusing total chick lens crystallins in the absence of urea, over the ampholyte range pH 3.5-10.

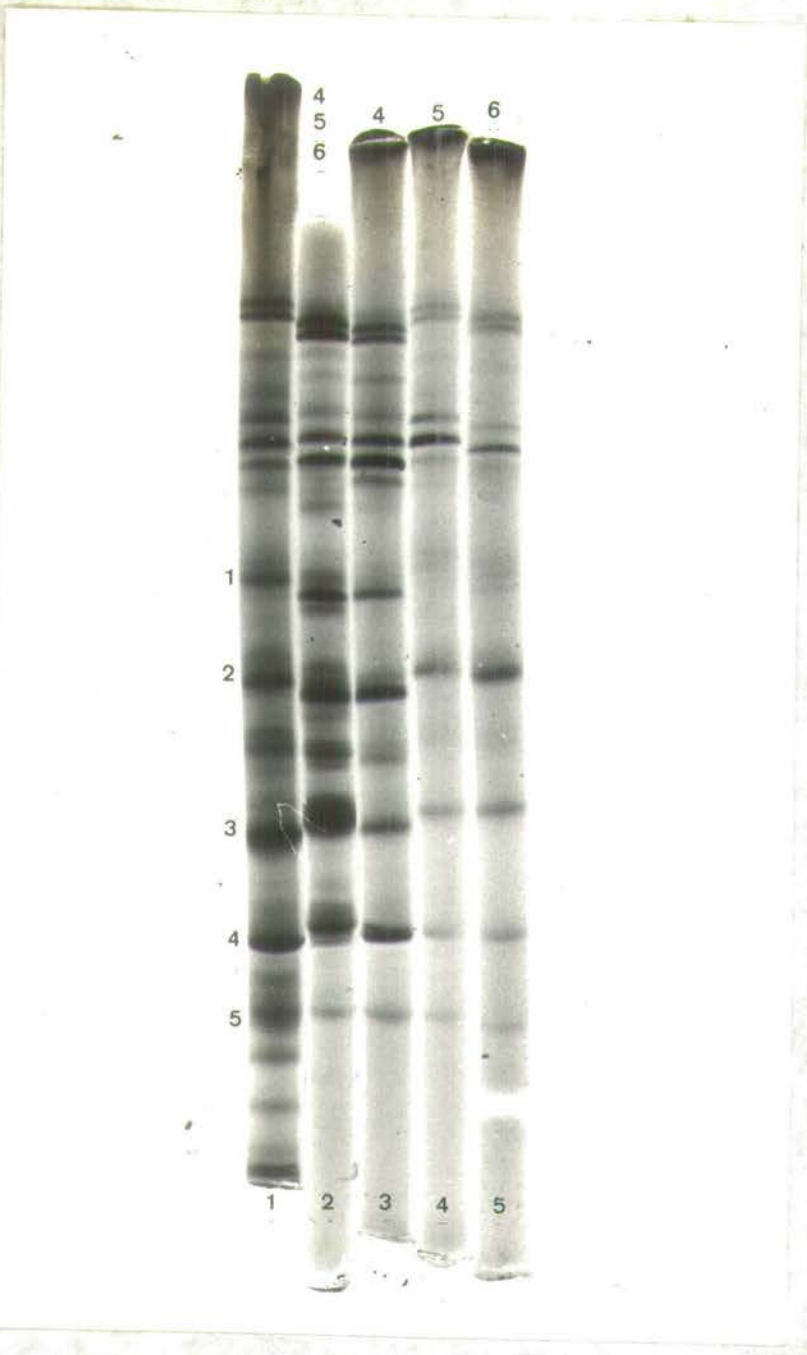


Figure 26. Re-electrofocusing in the presence of 6 M urea of the multiple band regions isolated by electrofocusing total chick lens crystallins in the absence of urea, Ampholine range pH 3.5 - 10 (see text). The numbers above the gels represent the band designations shown in figure 24. For comparison are shown a sample of total chick lens crystallins (250 µg), dissociated in 8 M urea prior to iso-electric focusing (gel 1) and the entire gel region that includes the fractions 4,5 and 6 (gel 2).

inherently more stable than others. The ratios and type of subunits in any particular complex may be determined by stereochemical restrictions. Alternatively but not exclusively, the complexes may reflect persistent linkages between certain subunits. Whatever criteria do, in fact, determine which assemblages are permissible, the final complexes appear subsequently to be sufficiently stable for routine isolation by gel electrofocusing.

The discovery that the polymers of high iso-electric point are composed predominantly of δ subunits, is at variance with the designations applied to the iso-electric focusing pattern of chick lens crystallins in thin-layer polyacrylamide gels, by Bours, (1971) who described them as "long line material" or beta-crystallins, (Bours, 1971). No evidence has been offered by the author, however, to support this designation (Bours and van Doorenmaalen, 1970; Bours, 1971). In fact, the iso-electric focusing pattern of only one purified protein fraction is presented, that of delta-crystallin (Bours and van Doorenmaalen, 1970). This fraction, obtained by ion-exchange chromatography on DEAE - Sephadex A-50, appeared to be slightly contaminated when tested by micro-immuno-electrophoresis and appeared as a complex of 7-8 components occuring in the pH range 4.9-5.3.

In chapter 5, the subunit analysis of a delta-crystallin fraction obtained by repeated gel filtration (Truman, 1968) was described. When thissample, which appeared to be immunologically pure was analysed by gel electrofocusing in non-dissociating conditions, several bands of high iso-electric point could be detected (figure 28). Four bands could be detected in the pH range 5.1-5.3 , whilst five bands occured in the pH range 6.5-8.0 . In addition, two further bands (probably components 9 and 10 of figure 24) could be detected with the Coomassie Blue stain. At low protein concentrations these components appeared very faint when stained with Fast Green, but all components were strongly represented in

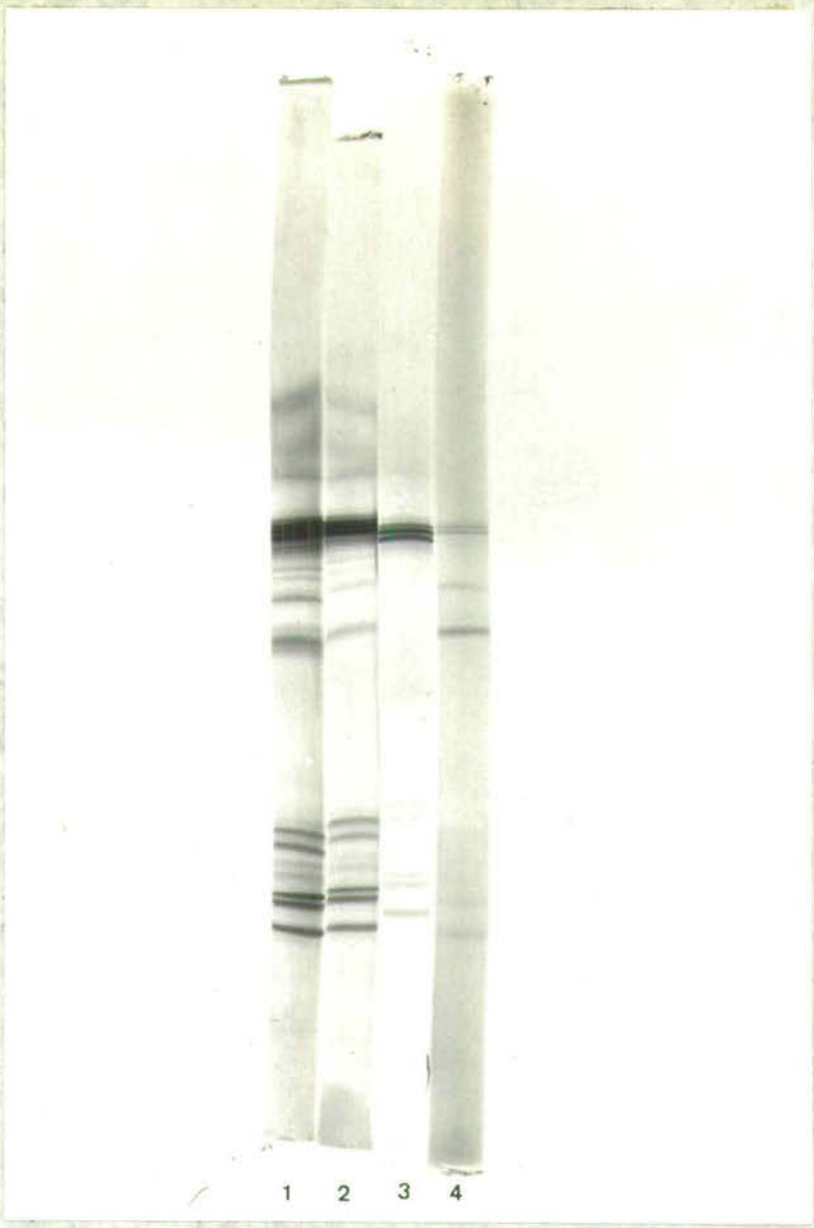


Figure 28. Iso-electric focusing pattern in the absence of urea of:

1) total chick lens crystallin (500 µg), stained with Fast Green
2) adult chick nucleus fraction (500 µg), stained with Fast Green.
3) chick delta-crystallin (100 µg), stained with Fast Green.
4) chick delta-crystallin (50 µg), stained with Coomassie Blue.

Ampholine range pH 3.5 - 10.

a delta-crystallin rich sample prepared from the nucleus of the adult chick lens (gel 2 figure 28).

The presence of these components of high iso-electric point in a purified fraction of delta-crystallin, analysed in non-dissociating conditions, confirmed the results obtained by subunit investigation, but cast doubt on the assertion that chick lens proteins from pI 5.68 to 7.58 were exclusively beta-crystallins or "long line material" (Bours, 1971).

The micro-heterogeneity revealed by iso-electric focusing of the immunologically homogeneous delta-crystallin sample has been attributed to differences in primary structure of the various protein components, however the possibility that the protein bands are composed of different proportions of subunits was not considered (Bours, 1971). Therefore it may be of value, at this point, to outline the exact significance of a focused band obtained in non-dissociating conditions. Iso-electric focusing is generally considered a comparatively mild process that does not cause decomposition of proteins (Vesterberg, 1970) (some immunological evidence is presented in a later section that suggests that no significant degree of denaturation occurs in gel electrofocusing of chick crystallins in non-dissociating conditions). However if no dissociative agents are present in the gel, it is to be expected, because of the aggregative properties of the crystallins, that many of the bands detected will represent complex polymers focused at their iso-electric point. The final iso-electric point of such complexes will depend on the proportions of the constituent monomers and also on the number of charged groups exposed within the aggregate. It is possible that subunit interactions within the polymer may lead to significant changes in the number of charged groups available, thus altering the overall net charge of the polymer. Even slight changes in the proportions of subunits within the polymer might

lead to different subunit interactions and separate iso-electric points. Secondly, for certain monomer proteins the iso-electric point in non-dissociating conditions appears to be dependent on the confirmation of the protein. Thus there is evidence that native bovine plasma albumin has seven buried basic groups in the uncharged state at its iso-ionic point (Williamson (A.H.) et al. 1973). When fully denatured the protein has an increased iso-electric point of approximately 0.7 pH unit. If for certain monomer proteins the folding of the polypeptide chain markedly affects its iso-electric properties, it is to be expected that a great number of separate focused bands will be detected in the analysis of polymers, where the variety of possible conformational forms and subunit interactions is greatly increased. Whilst the burial of uncharged amino-groups by the folding of the protein may be a comparatively rare event (Salaman and Williamson, 1973), more intricate conformational forms are possible in heteropolymers.

Iso-electric focusing of complex, aggregating proteins in non-dissociating conditions might then, in principle, be expected to produce focused bands reflecting the iso-ionic points of the various polymers, rather than those of homogeneous proteins. Where multiple samples of complex proteins such as blood serums or total wheat proteins have been investigated by combined gel electrofocusing and electrophoresis in a second dimension, many of the apparently single zones obtained by gel electrofocusing have been shown to consist of several components when further fractionated. Wrigley (1970) fractionated gliadin proteins by gel electrofocusing for a first dimensional separation. The unfixed electrofocused gel was then applied to a thin layer starch gel.

Migration in such a starch gel varies intensely with size and directly with charge at the pH of electrophoresis, exactly as in polyacrylamide electrophoresis.

Over 40 components could be detected, many of the single bands

obtained by gel electrofocusing showing as many as four or five components in the starch gel.

When serum proteins were first electrofocused in gels and then electrophoresed into 8% acrylamide slabs, the components which had been separated with high resolution in the first dimension, spread out the various bands over a large area, making the components easier to study (Dale and Latner, 1969). Again apparently single focused zones produced multiple components and many of the adjacent focused bands appeared to have components in common. Similar conclusions can be reached from an analysis of the two dimensional separation of serum proteins obtained by Kenrick and Margolis (1970). Here proteins were run from the electrofocusing gel into an acrylamide gel composed of a non-linear gradient of polyacrylamide ranging from 3.5 to 34 per cent, top to bottom. In addition when chromosomal proteins were fractionated by gel electrofocusing and the bands complexed with sodium dodecyl sulphate (SDS) prior to electrophoresis in a second dimension of polyacrylamide gel containing the detergent, single focused bands were shown to be made up of components of different molecular weights (MacGillivray and Rickwood, 1974).

Whilst these studies all revealed unexpected degrees of heterogeneity in the original protein samples, they emphasise the need to analyse each focused band, since it cannot be assumed that each band obtained in such conditions is pure or homogeneous. This conclusion also applies to protein peaks obtained through fractionation of samples by column electrofocusing. In the separation of guinea-pig serum proteins a third gamma-globulin could be detected by subsequent gel electrophoresis and densitometry, but was hidden by the serum albumin peak in the initial electrofocusing pattern (Stewart-Tull and Arbuthnott, 1971). Consequently a single peak in the elution pattern obtained with column electrofocusing cannot automatically be taken to indicate purity. In general, for multiple

samples, particularly strongly interacting proteins such as the lens crystallins, (see Introduction), it seems unlikely that a single method of fractionation, even the high resolution technique of iso-electric focusing in non-dissociating conditions, will produce a total separation of components.

Chapter 8

TESTS WITH ANTISERA TO TOTAL LENS PROTEINS SEPARATED BY GEL ELECTROFOCUSING IN NON-DISSOCIATING CONDITIONS

There are a considerable number of complications in elucidating immunological relationships between denatured proteins. Many denatured proteins become insoluble at their iso-electric point (Kleiner and Orten, 1966) making immuno-diffusion studies difficult. Although some authors have achieved immunological reactions with urea dissociated crystallins (Manski and Spector et al. 1972) all attempts to obtain precipitation lines with crystallins diffusing from urea-containing polyacrylamide using the antibodies available in this laboratory were unsuccessful. Most antibodies do in fact appear to be readily dissociated by urea (Clausen, 1969)with subsequent loss of specific reactivity. Moreover where the antibodies have been directed against a complex, non-dissociated protein the conformational properties of the protein subunits may be sufficiently altered in dissociating conditions to reduce or prevent immunological reaction.

Consequently the immunochemical reactions of protein antigens separated by gel electrofocusing in non-dissociating conditions were studied, in order to check the conclusions reached by subunit analysis of the various complexes isolated. In particular it was hoped to identify directly components of the beta-crystallin class. In addition it was also hoped to check the claim that in non-dissociating conditions of gel electrofocusing the proteins are arranged simply in the sequence alpha, delta and beta-crystallin in order of increasing iso-electric point (Bours 1971). An immuno-electrofocusing technique (Catsimpoolas, 1973) was employed in which the iso-electric focusing gel was embedded in buffered agar, then troughs were cut parallel to the gel and filled with antiserum (see Materials and Methods section).

The specificities and sources of the various rabbit antibodies used

Table 8. Antisera employed in immuno-electrofocusing studies.

Antiserum	Crystallin specificity	Reference
Anti-total lens proteins, Gallus gallus	α, β and δ	Clayton and Truman, 1974
Anti- " " " Rana pipiens	β	-
Anti- " " " Powan	β	-
Anti-chick lens IEF fraction	β	-
Anti- 1 (chick)	most anodal β-crystallin subunit	Clayton and Truman, 1974
Anti- 4-5 (chick)	intermediate β-crystallin subunit	" " " "
Anti- 9 (chick)	most cathodal β-crystallin subunit	" " " "
Anti-bovine	α	-

against the crystallin antigens after their iso-electric separation is shown in Table 8. The immunoprecipitin arcs formed during immuno-diffusion of each of these antibodies is shown in diaggrammatic form in figure 29. Unfortunately these immuno-electrofocusing studies were only at a preliminary stage when a severe infection killed off many of the antibody producing rabbits. However some important conclusions could still be reached, even on the basis of these preliminary results. For example, the immuno-electrofocusing pattern obtained with antisera prepared against total chick crystallins is remarkably complex (figure 29a). Clearly if the antigens were arranged simply in the sequence alpha, delta and beta-crystallins, from anode to cathode, in order of increasing iso-electric point, as claimed by Bours (1971) one would expect to obtain a much simpler pattern of three discrete immunological arcs. Because of the particular range of antisera available in the laboratory an attempt was made to identify both alpha and beta-crystallin immunological arcs.

Immuno-electrofocusing studies using antisera against beta-crystallins

The distribution of beta-crystallins as revealed by this technique is interesting. With a serum directed against the total crystallins of the frog, Rana pipiens, two immunoprecipitin arcs could be detected, distributed over most of the length of the ampholine arc, appearing to join only near the acidicend of the gel (figure 29b). Presumably this antibody is detecting two separate immunological determinants diffusing from the basic regions of the gel. Antibodies directed against the total crystallins of the Powan fish showed only a single immuno-precipitin arc of more limited length at the basic pH region of the gel (figure 29c). A slightly more extensive arc could be obtained with antibodies developed against a dissociated sample of chick lens cortex (rich in beta-crystallins), isolated by column electrofocusing and prepared by Dr. D. E. S. Truman (figure 29d). In contrast with antibodies prepared against a restricted range of subunits, prepared by gel filtration and urea

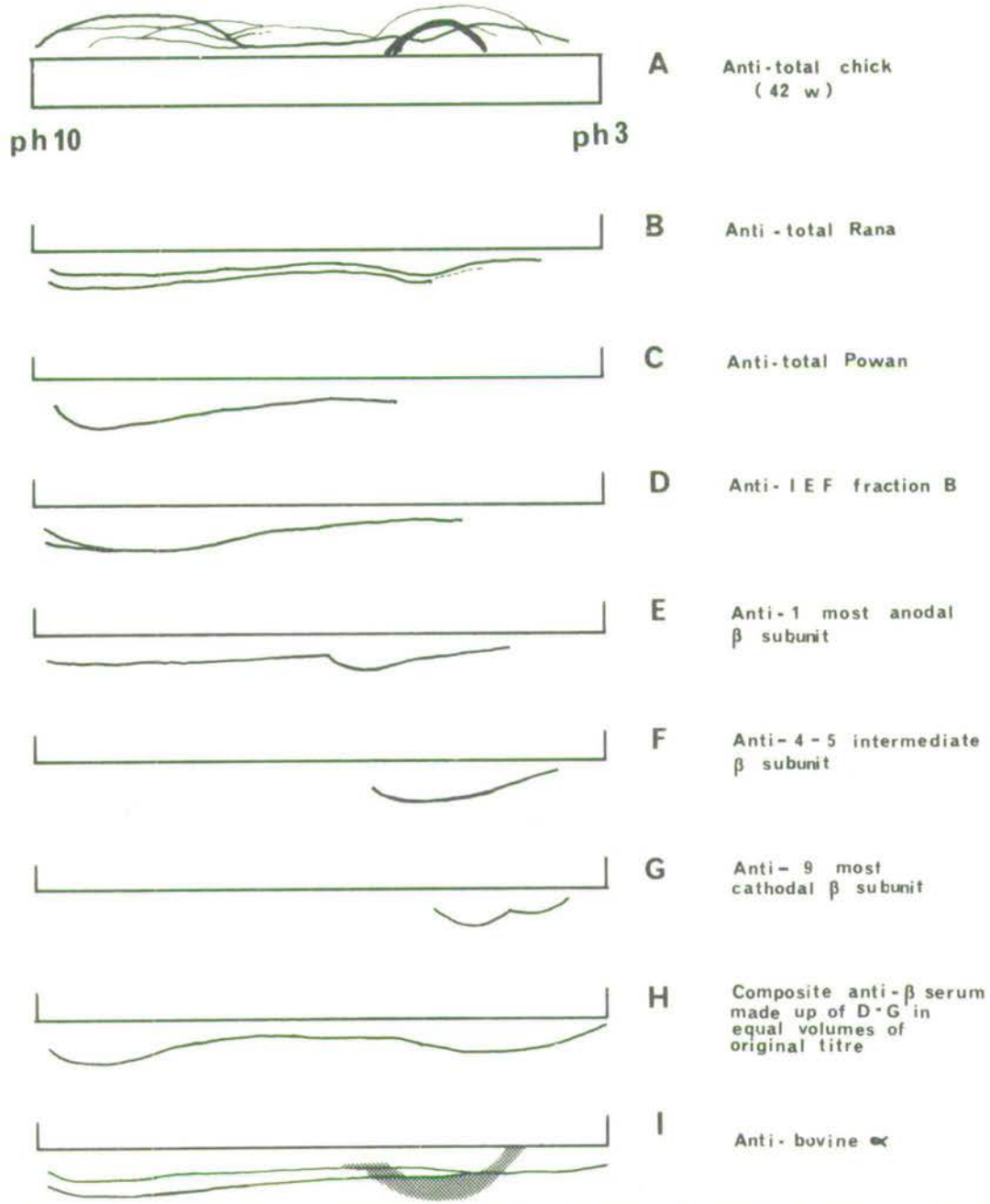


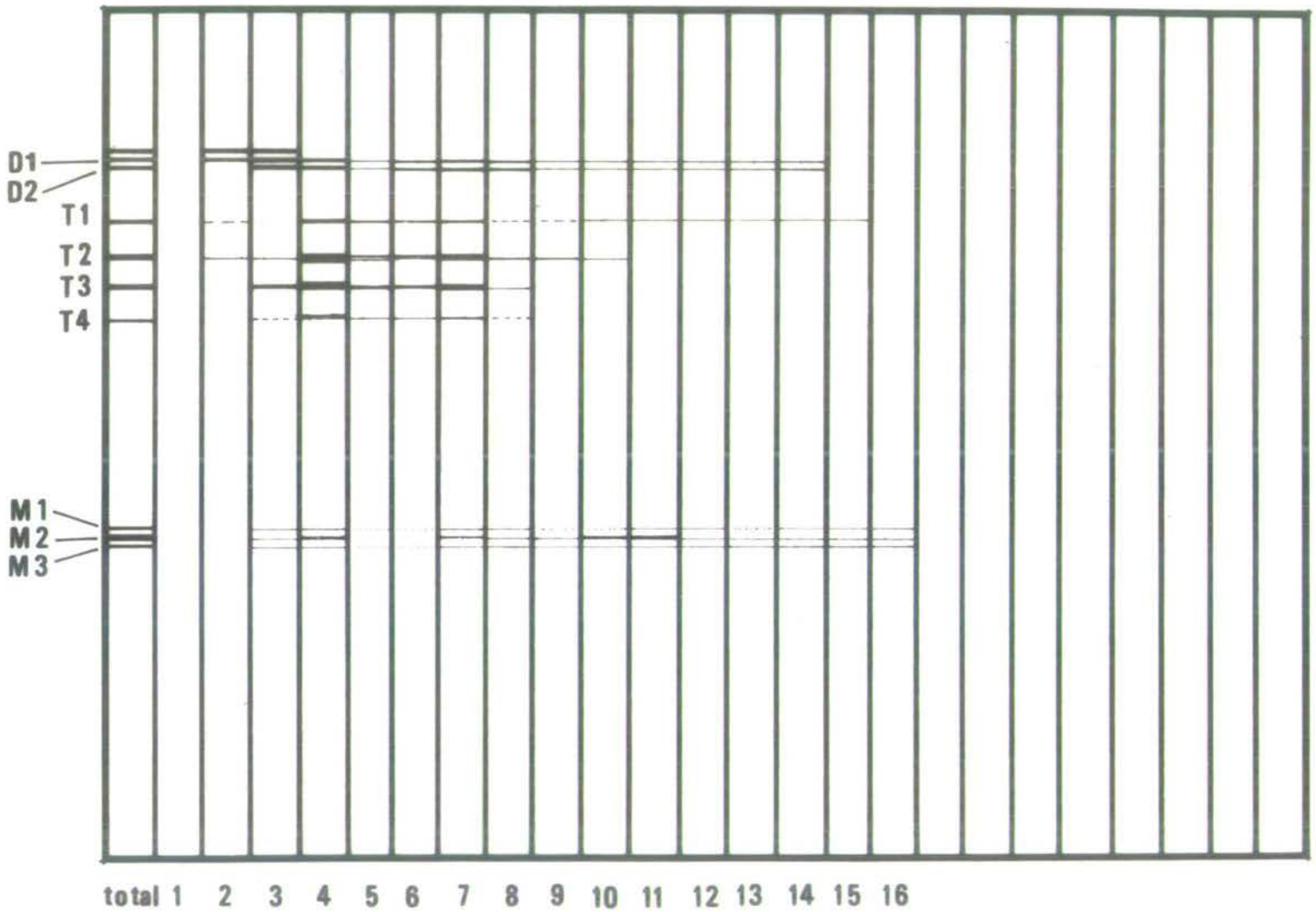
Figure 29. Diagrammatic representations of immunoelectrofocusing patterns. Total lens protein from adult chicks (at 500 µg per tube) was fractionated by gel electrofocusing in non-dissociating conditions over the pH range 3.5-10. The gels were then embedded in 1% agarose made up in ½ strength High Resolution Buffer (see Materials and Methods section). After 6 hours to allow diffusion of molecules from the polyacrylamide gel to the agarose, troughs were cut parallel to the gels at a distance of 8 mm from the edge of the gel, antiserum (approx .2 ml) introduced and immunodiffusion allowed to take place overnight in a humidity cabinet. After the immunoprecipitin arcs were stained (see Materials and Methods section) their patterns were carefully recorded by accurate drawing of the illuminated glass plates.

electrophoresis (Truman and Clayton, 1974; Clayton and Truman, 1974), the antisera produced single immunoprecipitin arcs that were restricted to only certain regions of the ampholine gel (figure 29 e.f.g.). However a composite anti-beta-crystallin serum made up to contain these three antibodies and the antisera directed against beta-crystallin fractions prepared by iso-electric focusing, gave a single immunoprecipitin arc that covered the entire length of the ampholine gel. Thus it appears that at least one immunological determinant is shared by the molecules making up the beta-crystallin class, as has also been show by the immuno-electrophoretic pattern obtained when total chick crystallins are tested with an antisera to amphibian lens (Clayton and Truman, 1974). In addition it appears that the beta-crystallin class can be found over the whole pH range of the ampholine gel and is not restricted to proteins of high iso-electric point, as suggested by Bours (1971). Other workers have also stressed the heterogeneity of iso-electric point exhibited by the chick beta-crystallins. When a purified preparation of beta-crystallins was separated by column isofocusing in sucrose, in non-dissociating conditions, the components were fractionated over the whole range pH3-8, i.e. no restriction to a high pH range was observed (Truman and Clayton, 1974).

However the restricted immunoprecipitin arcs obtained with antisera prepared against subunits appears anomalous if one considers only the electrophoretic mobility of the subunits and the corresponding immuno-electrofocusing patterns. For example the most cathodal subunit may be the lowest mobility subunit because it is the most positively charged subunit or the largest subunit, or (more likely) has the highest charge density.

It might therefore be expected to have a higher iso-electric point than the most anodal (fastest) subunit. Consequently one would expect the antiserum against this cathodal subunit (anti-9) to show an immunoprecipitin

Figure 30. Diagrammatic representation of the distribution pattern of beta crystallin subunits originally isolated in polymers obtained by gel electrofocusing total chick lens protein in non-dissociating conditions, over the pH range 3.5-10. The major components (numbered 1-16) were precipitated by 80% saturated ammonium sulphate, excised, and then analysed by gel electrofocusing in dissociating conditions. The distribution of these and other components were first shown in fig.



reaction with beta-crystallins diffusing from regions of high iso-electric point, if the original electrophoretic separation was determined mainly by the charge of the molecules. In practice, the reverse appears to be the case, the antisera reacting with antigens of low iso-electric point, (figure 29f). Similarly the antisera to the most anodal subunit reacts with antigens of high iso-electric point (figure 29g). If however one refers back to the subunit analyses of each of the fractions isolated by gel electrofocusing (figures 27,30), it can be seen that the major unassigned subunits, T_1, T_2, T_3 and T_4, hitherto suspected of being beta-crystallins, on the basis of re-running experiments (see figure 27, chapter 7) are only found in any quantity in fractions 2-8, derived from the region of the undissociated gel where the antisera (anti-9) and (anti-5) both give a restricted immunological arc. The only unassigned subunits present in any quantity in the cathodal regions of the gel are bands M_1, M_2 and M_3 (see figure 30) which appear to be found in various complexes throughout the whole pH range of the undissociated gel but are most strongly represented in fractions 10-16. Thus it is possible to reconcile the data from immuno-electrofocusing with that revealed by subunit analysis if one postulates that some determinants recognised by the two anti-sera (anti-9 and anti-4.5) are borne on the major subunits T_1, T_2, T_3 and T_4, whilst anti-sera (anti-1) is capable of detecting some determinants on the minor subunits M_1, M_2 and M_3, which have an extensive distribution in the ampholine gel. This could most plausibly occur if the slower subunits were separated in electrophoresis mainly on the basis of their molecular size rather than their charge. If this is the case then one can predict that the subunits T_1, T_2, T_3 and T_4 will have higher molecular weights than M_1, M_2 and M_3.

It is clear from the work of Clayton and Truman (1974) that some beta-

crystallin determinants can be shared by several subunits. The same determinant may be recognised on subunits of widely different electrophoretic mobilities or derived from aggregates of separate nature. Consequently it is easy to understand how a composite antisera derived from the various antibodies directed against isolated components of dissociated beta-crystallins can give a single immunoprecipitin arc, that covers the entire length of the ampholine gel.

Immuno-electrofocusing studies using an antiserum against alpha-crystallins.

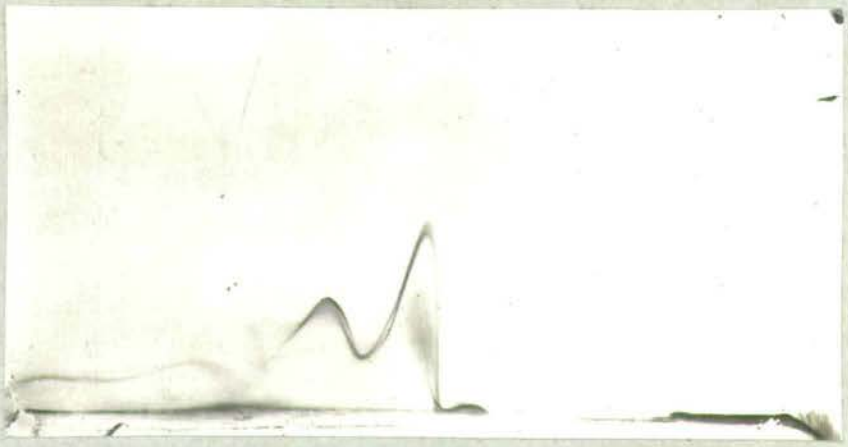
At the time of these experiments, only one antisera was available which had any enhanced specificity for alpha-crystallin. This was an antiserum made to a partially purified sample of bovine alpha-crystallin, so that although its main cross reaction could be expected to be with chick alpha-crystallin, it is also possible that it cross reacts slightly with chick beta-crystallins. One major wide arc could be detected in the pH 5 region of the gel, with a characteristic smeared appearance (figure 29i), this band almost certainly represents the main alpha-crystallin immunological reaction. It is just this region of the gel that was revealed by subunit analysis of the polymers obtained in non-dissociating conditions to contain large amounts of components 1 and 2, alpha-crystallin subunits (fractions 6-9, figure 27, chapter 7). In addition two minor, extensive arcs could also be detected, apparently meeting near the apex of the major arc. These lines may represent immunological reactions to beta-crystallins.

Electro-immunoprecipitation studies

The difficulties of using immunological procedures to analyse components obtained by urea dissociation were mentioned in Chapter 7. An attempt was made to develop a two dimensional technique similar to that described by Laurell (1965) and Clarke and Freeman (1968), since migration of components, but not urea, into the second dimension of agarose gel, should allow antibody reactions to develop. Using ampholine gels as the first dimension separation, only the components from the upper regions of the gel migrated into the agarose gel (figure 30A). The other components may not be soluble at their isoelectric point or possibly do not migrate under these conditions of pH. However a second buffer of pH 6.0 gave no improvement. These preliminary results remain interesting, however, because the upper portion of the gel gave a single immunological arc, separate from the continuous precipitin line formed from the alpha-crystallin subunits. Thus the components of lowest iso-electric point (D_1 and D_2), previously thought to be either delta or beta-crystallin components (see Chapter 6) can be finally classified as beta-crystallins. This result is not unreasonable, considering the close similarity in their iso-electric point to the beta-crystallin components T_{1-4}.

Difficulties were encountered with the differences in endosmosis between the polyacrylamide and agarose gels, but development of this technique, to increase the efficiency of identifying antigenically related components after the high-resolution separation of urea gel electrofocusing appears a promising and worthwhile task.

These results completed the classification of the major subunits revealed by gel electrofocusing in dissociating conditions. The final altributions are shown in figure 31.



alpha-1 alpha-2

Figure 30A. Antigen/antibody crossed electrophoresis of total chick crystallins after initial separation by gel electrofocusing in dissociating conditions (first dimension). The electrophoresis into the agarose gel containing antibody to total chick crystallins (second dimension) was performed as described in the Materials and Methods section. The origin of the electrofocusing gel was at the left. The peaks derived from the alpha-1 and alpha-2 subunits are arrowed.

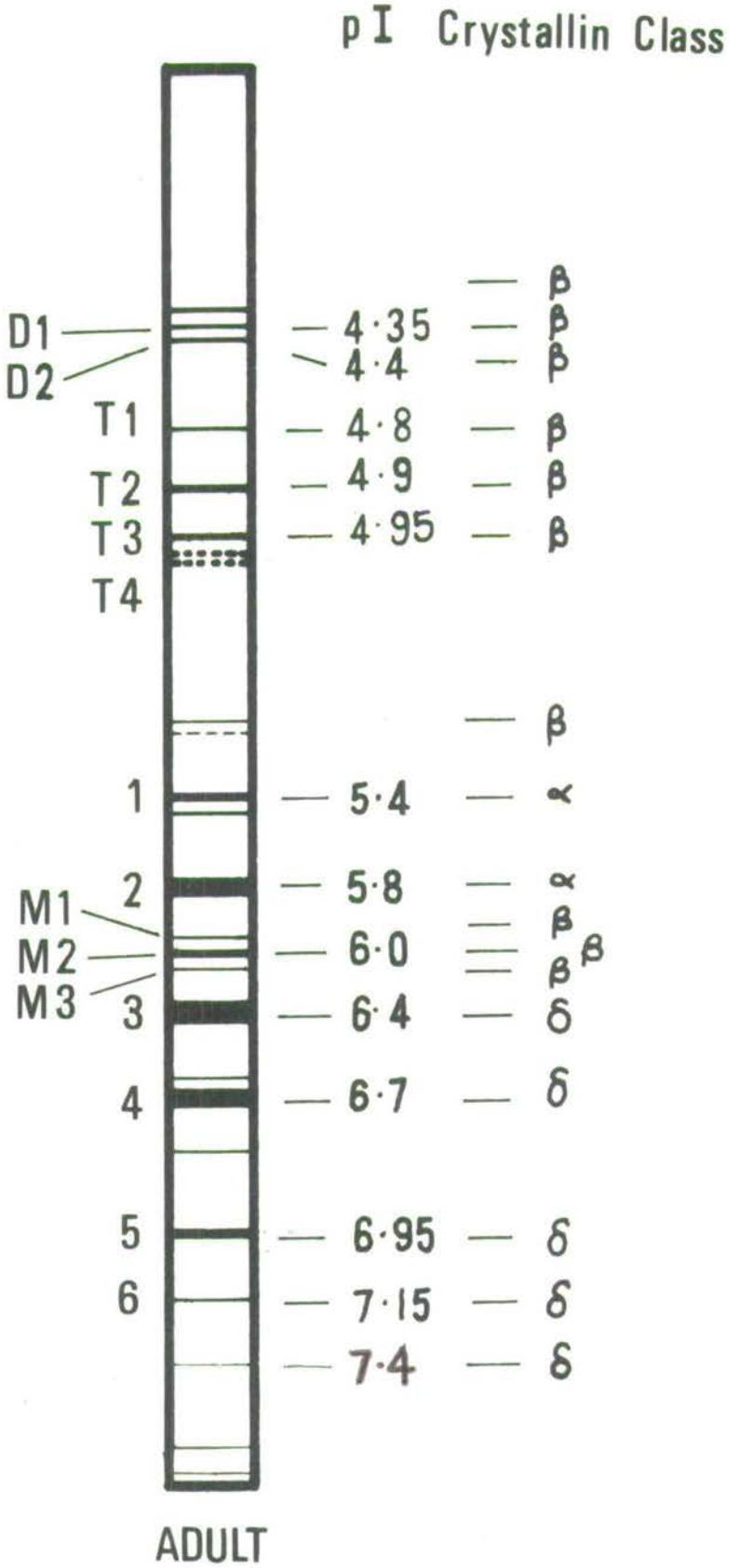


Figure 31. Diagram summarising the final assignation to crystallin class of the major subunits revealed by gel electrofocusing total chick crystallins in 6M urea. The pI of each major component is also indicated.

Chapter 9

Estimation of the range of molecular weights of the sub-units of the chick crystallins

The major proteins of the chick lens, the crystallins, are composed mainly of three classes of soluble proteins, differing in charge and in molecular weight (see Introduction). However, if as argued repeatedly the differentiation of the lens fibre is to be considered as a system involving the activity of a number of different genes, then it is the synthesis of the individual sub-units that must be followed. The enumeration and comparison of sub-units os obviously vital to any estimation of the number of related genes active in the tissue. Consequently it seemed of value to compare the number of sub-units revealed by gel electrofocusing in dissociating conditions with another high resolution technique, that of composite urea-sodium dodecyl sulphate polyacrylamide gel electrophoresis (MacGillivray et. al. 1972). This type of technique is a proven method for accurate molecular weight determinations for most polypeptides (Weber and Osborn, 1969). This was of additional advantage since published values for the molecular weights of the chick crystallin sub-units vary considerably:

The molecular weights of delta-crystallin sub-units

Piatigorsky et. al. (1972) and Craig and Piatigorsky (1973) obtained results indicating that the sub-unit molecular weight of delta-crystallin is about 45,000, by using electrophoresis in composite sodium dodecyl sulphate-polyacrylamide-agarose gels. Subsequent work by this group using both this technique and that of sedimentation analysis indicated that the sub-unit molecular weight of delta-crystallin was between 45,000 and 50,000 (Piatigorsky et. al. 1974). The embryonic protein analysed was not shown to be delta-crystallin

by immunological methods. The contention that the protein was delta-crystallin was made by direct comparison with the principal component found in mature lens fibre. In the first demonstration that delta-crystallin could be reduced in molecular weight by treatment with urea, Truman et. al. (1971) obtained a provisional value of 25,600 for the sub-unit molecular weight, using a gel filtration medium of porous glass beads.

The molecular weights of beta-crystallin sub-units

A polydisperse molecular weight ranging up to 59,000 was also estimated for chick beta-crystallin by gel filtration (Truman et. al. 1971). In addition molecular weight estimations in the presence of 7 M urea gave values of about 16,000 for the beta-crystallin sub-units. No other values appear to have been published. However; when a preparation of calf lens beta-crystallin was separated by gel filtration; the largest fraction was calculated to have a molecular weight of approximately 210,000 (Zigler and Sidbury, 1973). SDS-polyacrylamide electrophoresis of this fraction produced two major bands of 24,000 and 27,500 molecular weight together with two lesser components of molecular weight 31,000 and 35,000. The smaller fraction, molecular weight 52,000 yielded only two bands with molecular weights 24,000 and 27,500 when analysed in a similar fashion. Shapiro (1968b) eluted rabbit beta-crystallins from DEAE-cellulose and estimated the size of the native proteins as between 55,000 - 65,000 molecular weight, by calculating their sedimentation coefficients. Prolonged SDS-electrophoresis revealed a minimum of five different beta-sub-units in three different size classes of 21,000, 23,000 and 29,000, suggesting that each native complex consisted of a group of 2-3 poly-peptide chains.

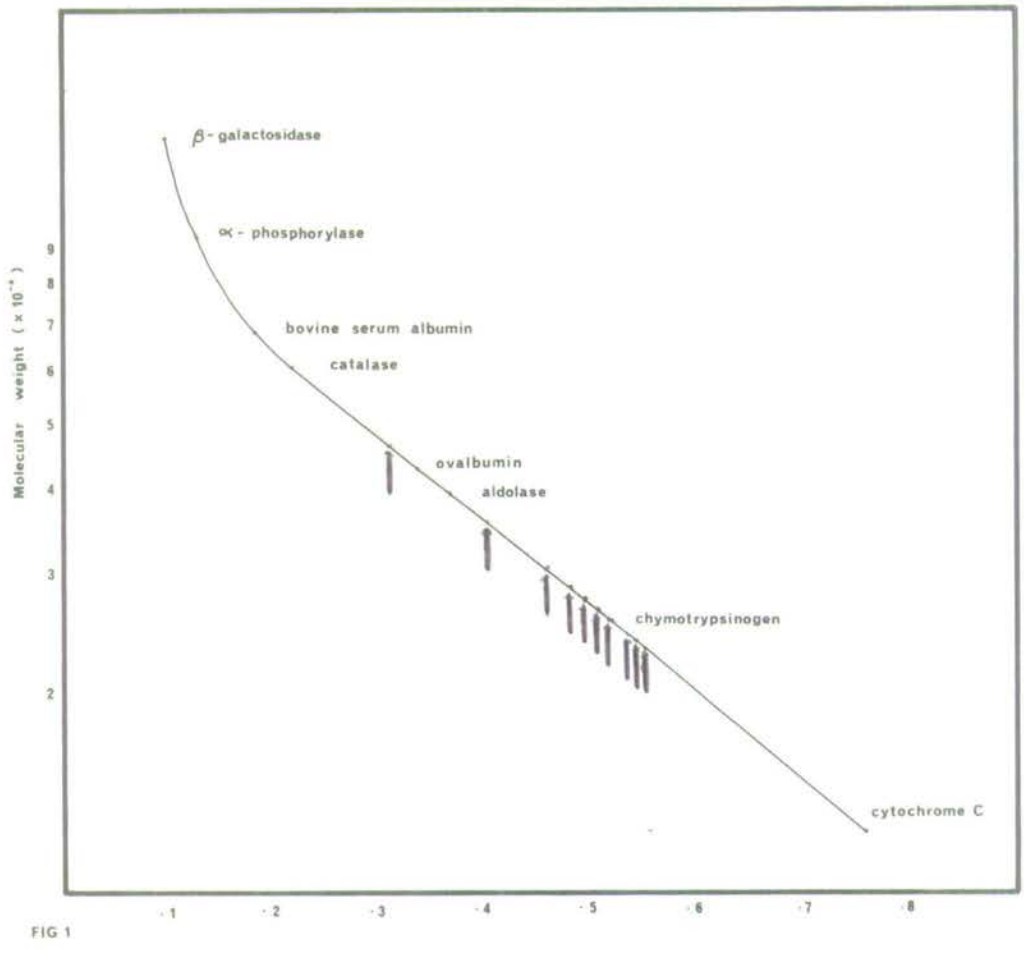


Figure 32. Estimation of the molecular weights of the subunits of the chick crystallins. The protein markers employed in the determinations were run on individual gels in the amounts and conditions specified in the Materials and Methods section. Mobilities were calculated according to the method of Weber and Osborn (1969). The arrows indicate the mobilities of the major protein subunits.

Because of these discrepancies in published values, it seemed of value to determine accurately the range of sub-unit molecular weights in a sample of the total lens protein of the adult chick, during the comparison of resolution obtainable with urea SDS-polyacrylamide electrophoresis and gel electrofocusing in urea. No attempt was made to identify directly any of the components separated by SDS electrophoresis in polyacrylamide gels.

Electrophoresis of Proteins from the Chick Lens on Composite Urea Polyacrylamide Gels in the Presence of Sodium Dodecyl Sulphate.

This technique was carried out exactly as described in the Materials and Methods section. The presence of urea ensures the complete dissociation of the protein during disaggregation by sodium dodecyl sulphate (MacGillivray et. al. 1972).

The calibration curve obtained by individually electrophoresing proteins of known sub-unit molecular weight in urea SDS-polyacrylamide gels is shown in Figure (32). The samples of total protein of the chick lens could be fractionated into ten major bands in a highly reproducible manner and their molecular weights estimated by comparing their mobilities with those of the marker proteins Figure (33a). The sub-unit molecular weights for these major components are listed in Table (9) in order of decreasing size. Above the predominant band of protein (molecular weight approximately 46,000) there appeared numerous (14 - 16) minor bands ranging in molecular weight from 50,000 to more than 150,000. The molecular weights of the more prominent of these minor bands are listed in Table (10a). In addition to these proteins four very faintly staining bands of low molecular weight could be detected, their sizes are listed in Table (10b).

Figure 33a. Sodium dodecyl-urea-polyacrylamide gel electrophoresis of total lens protein from adult chicks. Approximately 250 µg of lens protein were electrophoresed in the conditions described in the Materials and Methods section (gel 2). Also shown for direct size comparison are the electrophoretic patterns of several of the marker proteins: gel 1 catalase (MW, 60,000), gel 3 ovalbumin (43,000), gel 4 chymotrypsinogen (MW, 25,700).

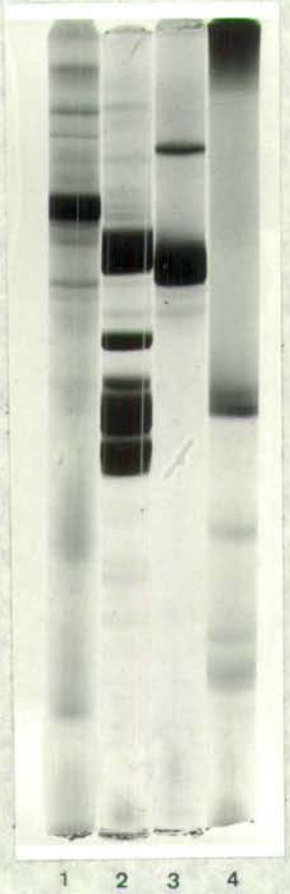


Figure 33b. Comparison of the resolution of adult chick lens protein available with urea-SDS-polyacrylamide electrophoresis or gel electrofocusing in urea. 250 µg samples of protein were electrophoresised in the conditions described in the Material and Methods section (gel on left) or electrofocused exactly according to the method described in Chapter 3. (gel on right).

The size of the principal component separated by this technique (molecular weight approximately 46,000) is in excellent agreement with other published values for the predominant protein found in mature chick lens fibres (Piatigorsky et. al. 1972; Craig and Piatigorsky, 1973; Piatigorsky et. al. 1974). Four of the other major bands have molecular weights almost identical to the four main components of calf lens beta-crystallin (Zigler and Sidbury, 1973). Thus there is excellent agreement about general size classes of sub-units within the crystallins when SDS-polyacrylamide gel electrophoresis is used. Only very minor bands could be detected in the molecular weight range of 16,000, the value obtained for chick beta-crystallin sub-units by gel filtration on glass beads (Truman et. al. 1971).

It seems clear that relative to SDS-electrophoresis in polyacrylamide gels, the glass bead filtration technique may underestimate molecular weights of crystallin sub-units. The discrepancy in reported differences in sub-unit molecular weights may be due to the particular filtration behaviour of the chick crystallins on porous glass beads. In particular Truman et. al. (1971) observed that the larger beta-crystallins were considerably retarded on the column in re-filtration. The authors pointed out that retardation may occur if adsorption phenomena occur or if there is any other type of interaction with the column medium. Any retardation of material would lead to an underestimate of sub-unit molecular weight. Whilst gel filtration techniques have been successfully used for the resolution of mammalian lens proteins (Testa, et. al. 1965) the general difficulty of fractionating the proteins of the chick lens has been pointed out by Truman (1968). There appears however to be a more general agreement of published values for the molecular weight of native delta crystallin (Table 11) as well as for the smaller size classes,

at least, of native beta-crystallins of various species (Table 12). It is possible that interactions between the chick crystallins and the glass bead filtration medium are increased in the presence of urea.

Comparison of Resolution of Chick Lens Protein Separated by Gel Electrofocusing in Urea or Urea-SDS-Polyacrylamide Electrophoresis.

Samples (250 µg) of the lens proteins of adult chicks were fractionated either by Gel electrofocusing in urea or by urea-SDS-polyacrylamide electrophoresis, then stained with 0.2% Coomassie Blue in identical fashion, exactly as described in the Materials and Methods section. In SDS-electrophoresis ten major bands were obtained in a closely compacted fashion, distributed over a region of about 3-5 centimetres (Figure 33b). In contrast, in gel electrofocusing fourteen major bands could be detected, well distributed over the whole length of the gel (Figure 33b). Clearly the latter technique offers advantages in isolating individual components, slicing gels for radioactive counting etcetera. As expected if resolution is poorer, the major components separated on the basis of their molecular weight stain more heavily than major components isolated by virtue of their iso-electric point. The wide, diffuse nature of the predominant component found in SDS-electrophoresis of chick lens proteins may, possibly, reflect a heterogeneous population of proteins, incompletely resolved by this particular technique.

About fourteen minor components can be detected with the gel electrofocusing method whilst between fourteen and sixteen components, all of high molecular weight, can be stained to a similar level of intensity after SDS electrophoresis. Some of these components may be active specifically in the youngest epithelium, since some components of high molecular weight are known to be synthesised at a

much higher rate in the central epithelium compared to either the central epithelium or the fibre body (Piatigorsky et. al. 1972).

In addition the electrophoretic technique also separated five very faint components in the low molecular weight range (Figure 33a and Table 10b), compared to perhaps three similarly faint components isolated by gel electrofocusing.

In general then, either technique will resolve something like thirty components in a sample of lens protein from adult chicks: however the additional resolution of major components favours the use of the electrofocusing method, as does their more widespread distribution.

Table (9)

The Molecular Weights of the Major Protein Sub-units of the Chick lens, as determined by urea-SDS polyacrylamide gel electrophoresis.

Sub-unit	Mobility	Sub-unit Molecular Weight
A	0.305	46,000
B	0.403	35,000
C	0.452	30,500
D	0.472	29,000
E	0.482	28,000
F	0.492	27,000
G	0.511	26,000
H	0.536	24,000
I	0.545	23,500
J	0.550	23,000

Table (10)

The Molecular Weights of some minor components of the Chick Lens, as determined by urea-SDS polyacrylamide gel electrophoresis:

Table 10(a) Proteins of High Molecular Weight.

Distance Migrated (mm)	Mobility	Molecular Weight
0.7	0.069	140,000
1.0	0.098	130,000
1.7	0.167	73,000
2.05	0.201	63,000
2.15	0.211	61,000
2.3	0.226	59,000
2.4	0.236	57,000

Table 10(b) Minor Components of Low Molecular Weight.

Distance Migrated (mm)	Mobility	Molecular Weight
6.4	0.629	18,500
6.9	0.678	16,000
7.2	0.708	14,700
7.7	0.757	12,500

Table (11)

Values obtained for the Molecular Weight of Native Chick Delta Crystallin

Analytical Technique	Molecular Weight	Reference
Sedimentation Velocity	200,000	Maisel and Langman (1961a)
Sedimentation Coefficient	150,000	Hoenders (1965) quoted in Truman et. al. (1971)
Two Dimensional Polyacrylamide Electrophoresis	150,000-165,000 (4 proteins) 460,000 (1 protein)	Zwaan (1968)
Gel Filtration	150,000-158,000	Truman et. al. (1971)
Sedimentation Velocity	200,000	Piatigorsky et. al. (1974)
Gel Filtration	215,000	

Table (12)

Values obtained for the Molecular Weight of Native Beta Crystallin of Various Species

Analytical Technique	Species	Molecular Weight	Reference
Sedimentation Coefficient	Rabbit	55-65,000	Shapiro (1968b)
Gel Filtration	Chick	59,000	Truman et. al. (1971)
Gel Filtration	Calf	52,000 and 210,000	Zigler and Sidbury (1973)

Chapter 10

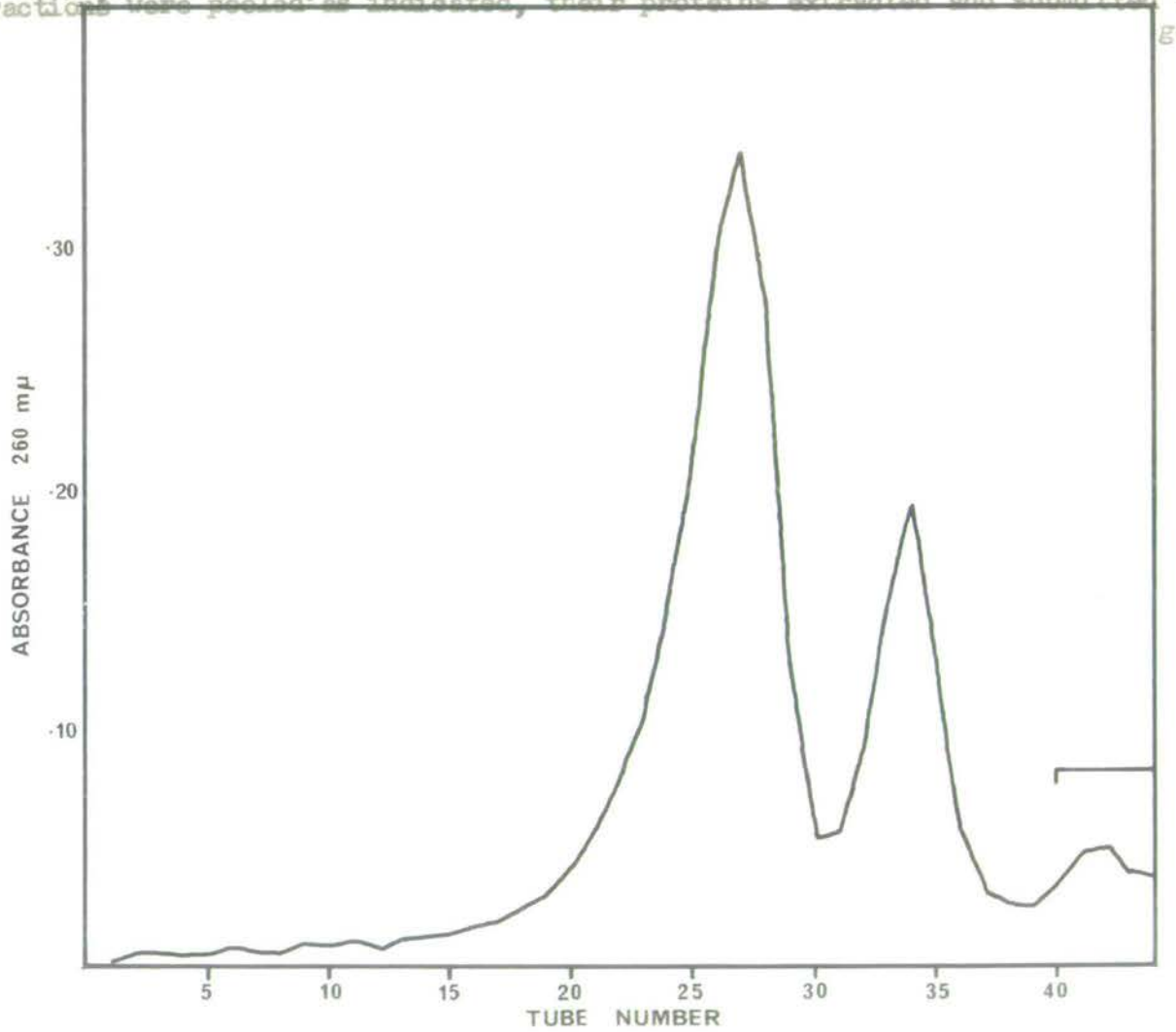
Comparison of the protein moiety of chick lens mRNP with the proteins removed from polyribosomes by treatment with KCl or Sodium Deoxycholate

Olsnes (1970) showed that when polyribosomes from rat liver were treated with 1% sodium deoxycholate or 0.5 M KCl. the buoyant density of the rapidly labelled RNA in caesium chloride gradients was considerably ~~reduced~~ increased. In contrast the buoyant density of the ribosomal sub-units was only slightly affected. The results indicated that these treatments could remove a substantial part of the protein associated with the rapidly labelled RNA without bringing into solution many structural proteins from the ribosomes. A similar release of protein from the rapidly labelled RNA of polysomes could not be obtained by the use of the non-ionic detergent Triton X-100.

The proteins removed from the rat liver polyribosomes were further characterised in a later report (Olsnes, 1971c) and four main protein components were revealed by polyacrylamide-gel electrophoresis. Only one of these components, of molecular weight 160,000 co-sedimented with the rapidly labelled RNA after the polysomes were dissociated with EDTA. When EDTA-treated ~~rabbit~~ reticulocytes of rabbits were subjected to sucrose gradient centrifugation, a very similar protein was found in bulk in the region of the gradient in which the mRNP complex was expected to sediment. (However a full confirmation that this region did indeed contain the 14s mRNP for rabbit globins was not carried out.)

The results indicated that the proteins attached to the mRNA in both rat liver polysomes and those of rabbit reticulocytes appeared to have very similar characteristics. Furthermore they indicated that such proteins may well be selectively removed by treatment with

Figure 34. Sucrose gradient centrifugation of chick lens polysomes treated with EDTA. A post mitochondrian supernatant from lenses of day old chicks was layered over 0.5 M sucrose in medium A and centrifuged overnight, for the preparation of polysomes in low ionic conditions in the 3 x 20 rotor head of the MSE superspeed centrifuge at 25,000 rev./mm., at 4°C. The polysomes were divided into two parts each of approximately 3 A_{260} units and made up to contain 33 mM EDTA. The polysomes were then layered onto 10-30% sucrose gradients made up in 50 mM Tris (pH 7.4 at 20°C). The gradients were centrifuged in the 3 x 20 rotor head, of the MSE superspeed centrifuge, for 6.5 hrs. at 30,000 rev/min., at 4°C. 15 drop fractions were collected and their absorbance at 260 m.u determined. Selected fractions were pooled as indicated, their proteins extracted and submitted to SDS polyacrylamide gel electrophoresis.



sodium deoxycholate or high salt concentrations.

Accordingly it was of interest to compare the proteins removed from chick lens polysomes by these treatments with those proteins found in the position of the lens mRNP in sucrose gradients. It was hoped that such an experiment would determine directly whether any selective enrichment of specific RNA-bound proteins could be obtained by these polysome treatments. Secondly, by characterising the mRNP from chick lenses initially in low salt buffer, the degree of non-specific interactions possible between the RNA and proteins could be eventually estimated, by a comparison with the mRNP obtained in ionic conditions which minimise artefactual associations.

Many recent studies have indicated that when polyribosomes are treated with EDTA the ribosomal sub-units are dissociated and complexes containing rapidly labelled RNA and protein are liberated (Burny et. al. 1969, Lebleu et. al. 1971, Williamson et. al. 1971). When polyribosomes from chick lenses were treated with 33 mM EDTA and submitted to sucrose gradient centrifugation, the large and small ribosomal sub-units (revealed by their absorbance at 260 nm) sedimented as two discrete peaks (Figure34). A third minor peak (containing less than 7% of the total 260nm absorbance) sedimented in the lower portion of the gradient. This broad zone was presumed to contain the chick lens mRNP complex since similar fractions showed template activity in cell free systems (Clayton and Truman, unpublished results). The proteins from this fraction of the gradient were then extracted and compared to the proteins removed from the polyribosomes by treatment with KCl or sodium deoxycholate (see Materials and Methods section). These two fractions are referred to respectively as the "high salt wash" fraction and the "deoxycholate wash" fraction. In figure(35) are shown the

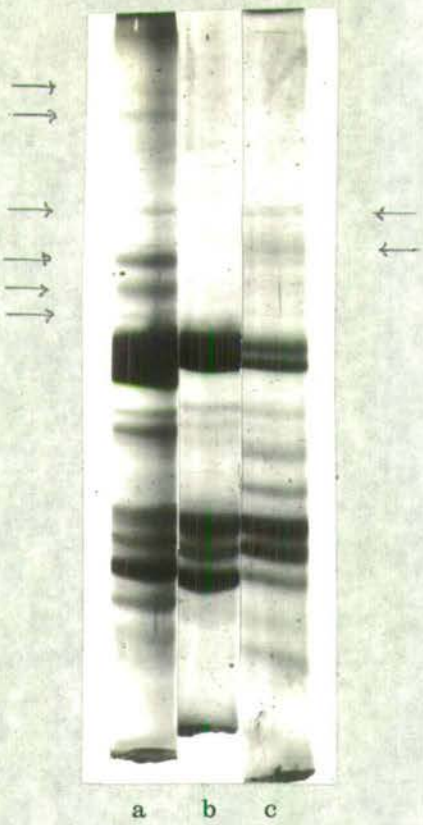


Figure 35. Comparison of the proteins associated with the putative mRNP fraction of chick lens polyribosomes with the proteins removed from the polyribosomes by treatment with KCL or sodium deoxycholate. The polyacrylamide gel electrophoresis in the presence of SDS was carried out in 11 x 0.6 cm glass tubes and the gels stained with amide-black. All other conditions were as described in the Materials and Methods section. The arrows indicate components of high molecular weight discussed in the text. Loadings (approximate): a) putative mRNP fraction 50 µg b) deoxycholate wash of polysomes 40 µg c) 0.5 M KCL wash of polysomes 40 µg

protein patterns obtained after electrophoresis of the various fractions in 15% polyacrylamide gel containing 4M area and 0.1% SDS.

The most striking feature of the comparison is the similarity in the protein patterns of all three preparations, particularly in the lower region of the gels (molecular weight range 50,000 - 20,000). Above this region however components of high molecular weight were present in any quantity only in the lens mRNP fraction although they represented only a minor portion of the total protein pattern revealed by the electrophoresis. In the lens mRNP sample 6 bands (arrowed) were apparent in the upper portion of the gel, of which possibly two (arrowed) were represented in the "high salt wash" as judged by similar electrophoretic mobility. No corresponding bands were detected in the"deoxycholate wash fraction". However comparison of these preparations with that of a sample of total lens proteins of adult chickens suggested that many of the lower molecular weight components represented lens crystallins. When the "high salt wash fraction" was explicitly compared to total lens crystallins, a remarkable number of major components appeared to have similar electrophoretic mobilities (see figure 41, chapter 1). This subsequent analysis also revealed that none of the components of high molecular weight found in the "high salt wash" fraction were common to mRNP fractions. Olsnes (1970) has shown that the main protein components released by 0.5 M KCl were very similar to those released by sodium deoxycholate but did not compare these preparations with a sample of total cytoplasmic protein from rat liver cells.

The bulk of material released by the polysome treatments and found attached to the presumptive mRNP particle thus appeared to consist of lens crystallins. Clearly in the ionic conditions used much of the

protein bound to the lens mRNA consisted of cytoplasmic protein adsorbed during the homogenisation. Furthermore the polysome treatments were clearly releasing the crystallins in bulk from the ribosomes, together with many other minor components. Obviously though, there was no significant enrichment of specific RNA-bound proteins obtainable by the polysome treatments. In a study on the mRNP of KB cells, Kumer and Lindberg (1972) compared the sedimentation rate and buoyant density of such mRNP when polysomes were prepared in isotonic and high salt buffers. The buoyant density analysis revealed that the mRNP from polysomes prepared in high salt buffer contained about half the protein composition compared to the mRNP prepared in isotonic buffer. The authors suggested that polysomal mRNP might contain two different groups of proteins: one weakly bound and easily removed by a high salt wash (0.5 M KCl) and a second group of proteins tightly bound. Consequently when attempting to minimise artefactual associations by employing hypertonic buffers there appeared a possibility that proteins bound specifically but weakly to mRNA might be lost. However the finding that 0.5 M KCl treatment of lens polysomes was primarily removing cytoplasmic proteins (crystallins) from the ribosomes, without removing to any degree the high molecular weight proteins found in the mRNP region of sucrose gradients, suggested that high salt washes of polysomes might cut down significantly the amount of cytoplasmic protein adsorbed during cell fractionation, without the loss of possibly crucial proteins with weak binding properties. Blobel (1972) used a high salt wash of polysomes from rabbit reticulocytes to investigate the proteins tightly bound to mRNA. The possibility that other major proteins of interest were lost because they were only weakly attached to the mRNA appears to be ruled out by the results reported here.

Chapter 11

Characterisation of the Messenger Ribonucleoproteins obtained by Zonal Centrifugation of Chick Lens Polyribosomes.

Zonal ultracentrifugation of EDTA-treated chick lens polysomes was shown by Williamson et. al. (1972) to result in the release of two ribonucleoproteins sedimenting at approximately 15s and 19s. The RNA from these regions were added to cell-free systems derived from either duck reticulocytes or Landshutz ascites cells. In both systems the added lens RNA stimulated incorporation into proteins which could be specifically precipitated with anti-bodies directed against chick lens proteins. The proteins made in the *in vitro* system co-electrophoresed with unlabelled carrier lens proteins and showed a similar pattern of activity to control electrophoretic profiles of crystallins labelled by incubating the lenses of one day post-hatch chicks with ^{14}C amino-acids. With this isolation and identification of the chick lens mRNA by established methods it appeared feasible to determine whether the protein components of the two messenger ribonucleoproteins were identical. This was attempted by using the same isolation techniques but employing high salt washed polyribosomes to minimise contamination of the mRNP particles by non-specific cytoplasmic proteins.

The intial technique (described in the Materials and Methods section) for the isolation of high salt washed polysomes was employed in this attempt to isolate lens mRNP. The zonal ultracentrifuge profile obtained with these high salt washed polysomes after EDTA treatment is shown in Figure (36). In this particular preparation the putative mRNP fraction was isolated as a single peak. This fraction was precipitated as described in the Material and Methods section. One aliquot was utilised for the isolation and electrophoresis of the RNA moiety, a second aliquot was subsequently used for the analysis

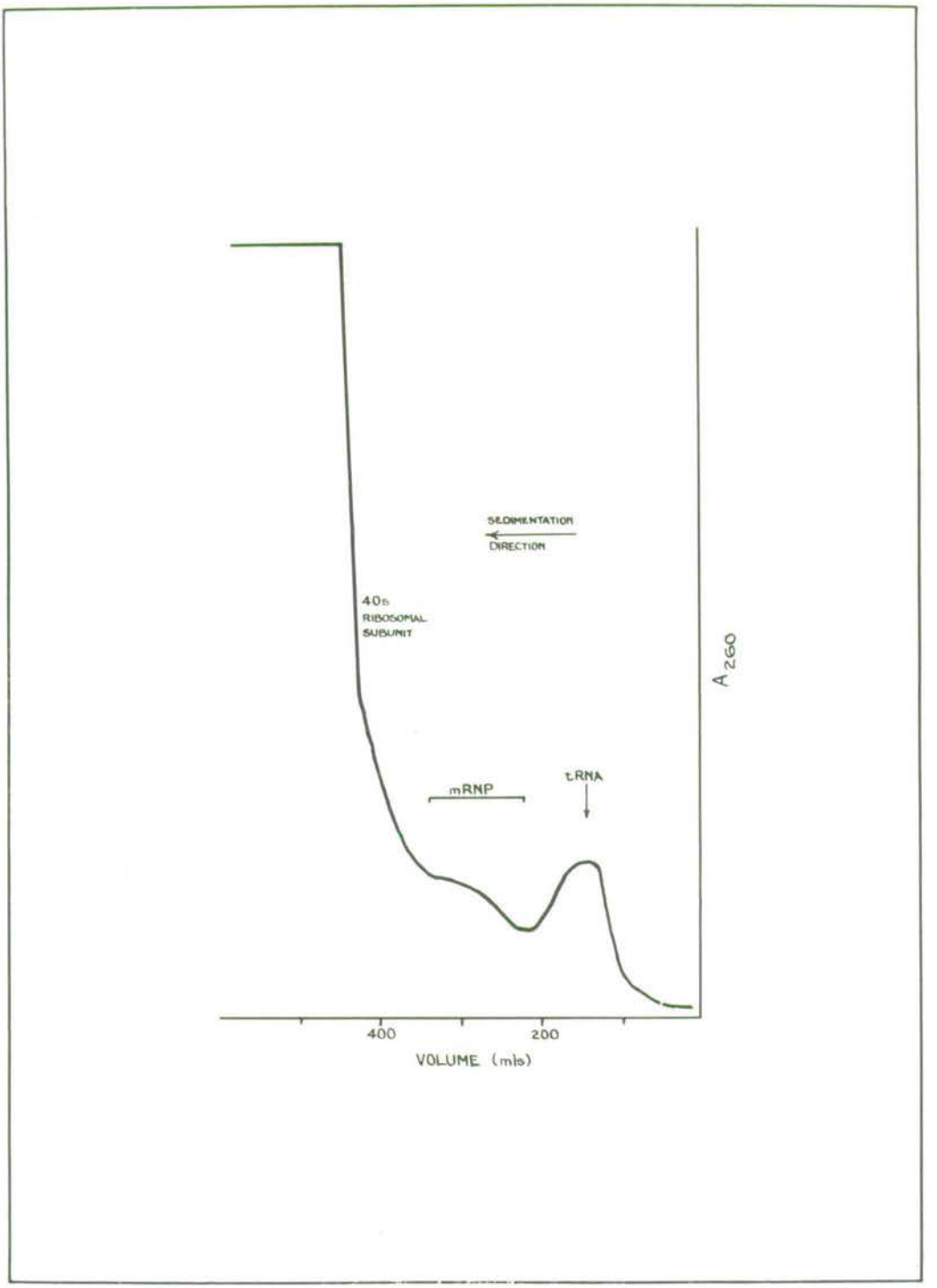
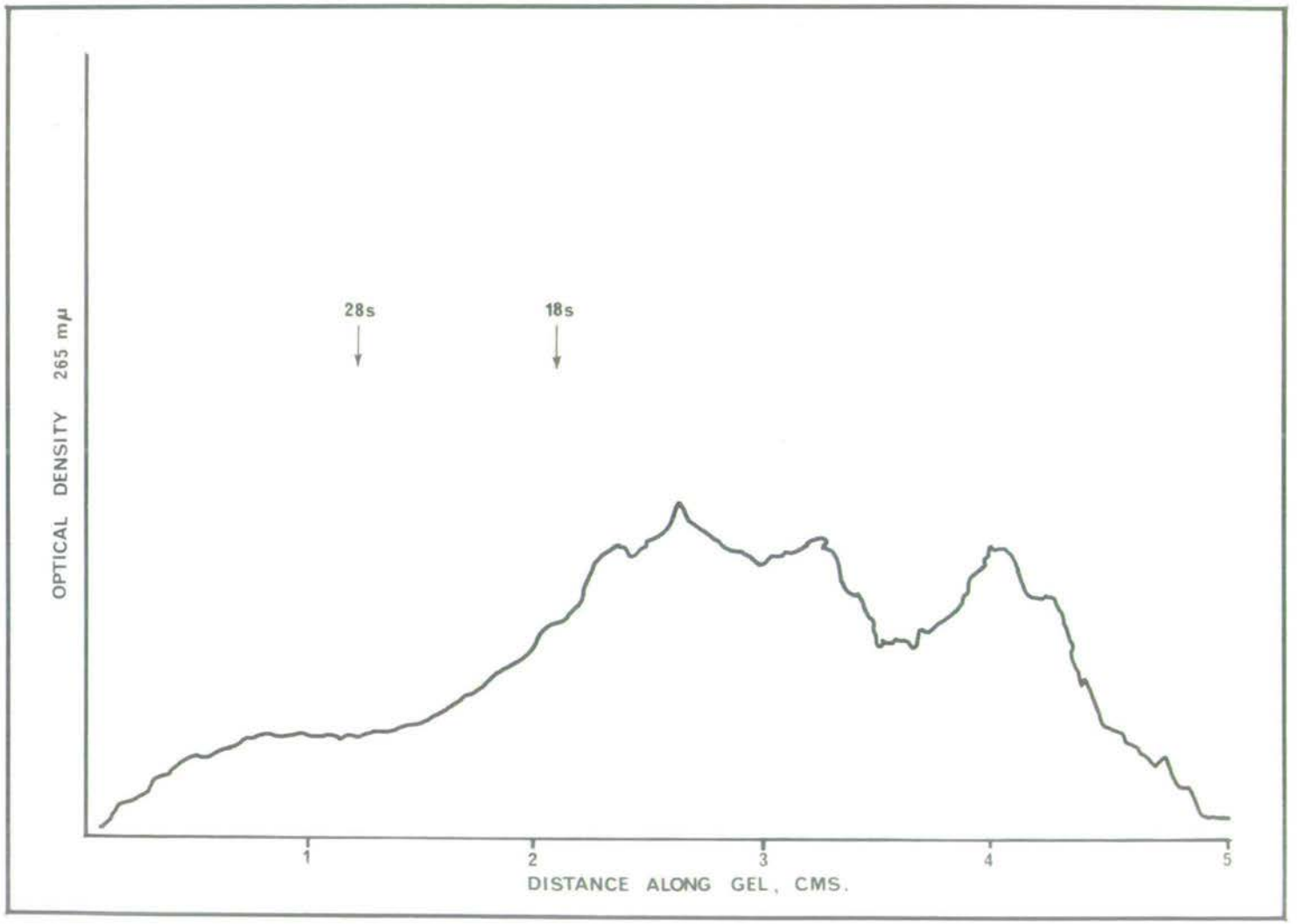


Figure 36. Zonal ultracentrifugation of EDTA-treated chick lens polysomes. Approximately 400 A_{260} units of high salt washed polysomes were dissociated with 33 mM EDTA and fractionated on a 10-40% (w/v) convex sucrose gradient in 0.02 M Tris, pH 7.4, in a sample volume of 13 ml of 3% sucrose. The overlay consisted of 80ml of 0.02 M Tris, pH 7.4. Centrifugation was at 37,000 rev./min. for 16 hours at 4°C in the B-XIV zonal rotor (M.S.E. Ltd., Crawley). The absorbance at 260 nm was read out through a SP-800 (Unicam) spectrophotometer with a Perkin-Elmer variable path length UV flow cell, amplified 25 x with a servoscribe chart recorder. The fraction taken is indicated in the diagram by bars.

Figure 37. 2.6% polyacrylamide gel electrophoresis of RNA samples isolated from ribonucleoprotein fractions obtained by EDTA-dissociation of polysomes. Migration from left to right, gels 7.5 cm long and 0.63 cm diameter, electrophoresis at 2.5 mA/gel for 20 min. and then 5 mA/gel for 90 min. at 20°C. The electrophoresis buffer consisted of 36 mM Tris, 30 mM Na phosphate, ImM EDTA, 0.2% SDS, pH 7.8. Other conditions as Williamson et. al. (1971) and Loening (1967). Loading of RNP sample -0.2 A_{260} units. For comparison the position of lens ribosomal 28s and 18s RNA is also shown.



of the protein moiety of the RNP. Approximately 6 A_{260} units of RNA, 1.5% of the total polysomal RNA, were recovered in this fraction. The polyacrylamide gel electrophoresis pattern of the RNA isolated from the EDTA-dissociated ribonucleoprotein fraction is shown in Figure (37). The RNA showed considerable heterogeneity with three clear peaks. However this gel electrophoretic pattern is in excellent agreement with the diagram (figure 2) published in Williamson et. al. (1972) where the mRNP was separated into two peaks by the initial zonal centrifugation. The RNA ranged in size from 22s to 4s, with three peaks at 15s, 12s, and 9s. The close correspondence in electrophoretic pattern of this RNA preparation with those of samples of demonstrated messenger activity suggested that the fraction isolated did contain the mRNP(s) for the lens crystallins.

The analysis of the protein moiety of the putative mRNP particle was carried out by polyacrylamide gel electrophoresis in the presence of SDS, as described in the Materials and Methods section, revealing three discrete high molecular weight components and three diffuse components in the 35,000 - 20,000 molecular weight range (figure 38). It appeared that although the lens synthesises many different proteins, the polysomal mRNA species were associated with comparatively few tightly-bound proteins. As shown subsequently the lower molecular weight components varied considerably from preparation to preparation and presumably were contaminants that adsorbed or co-sedimented with the mRNP particle.

The zonal centrifugation of EDTA treated polyribosomes from day old chick lens was repeated in order to confirm that the same set of proteins could be isolated routinely from the putative mRNP particles. Furthermore it was necessary to achieve a greater degree of separation within this mRNP region, in order to ascertain whether discrete polysomal

Figure 38 Polyacrylamide electrophoresis in the presence of SDS of the proteins associated with the putative mRNP region of chick lens polyribosomes. The polyribosomes were washed in 0.5 M KCL prior to being dissociated with EDTA. The sample (in 100 µl) was obtained from 0.3 A_{260} units of RNP. The length of the polymerised gel prior to electrophoresis was 9.3 cm. Gels were stained with Coomassie Brilliant Blue for 30 minutes. All other electrophoretic conditions were as described in the Materials and Methods section.

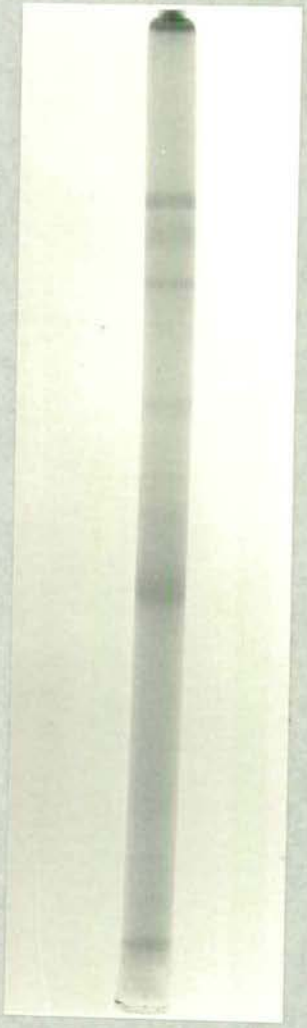
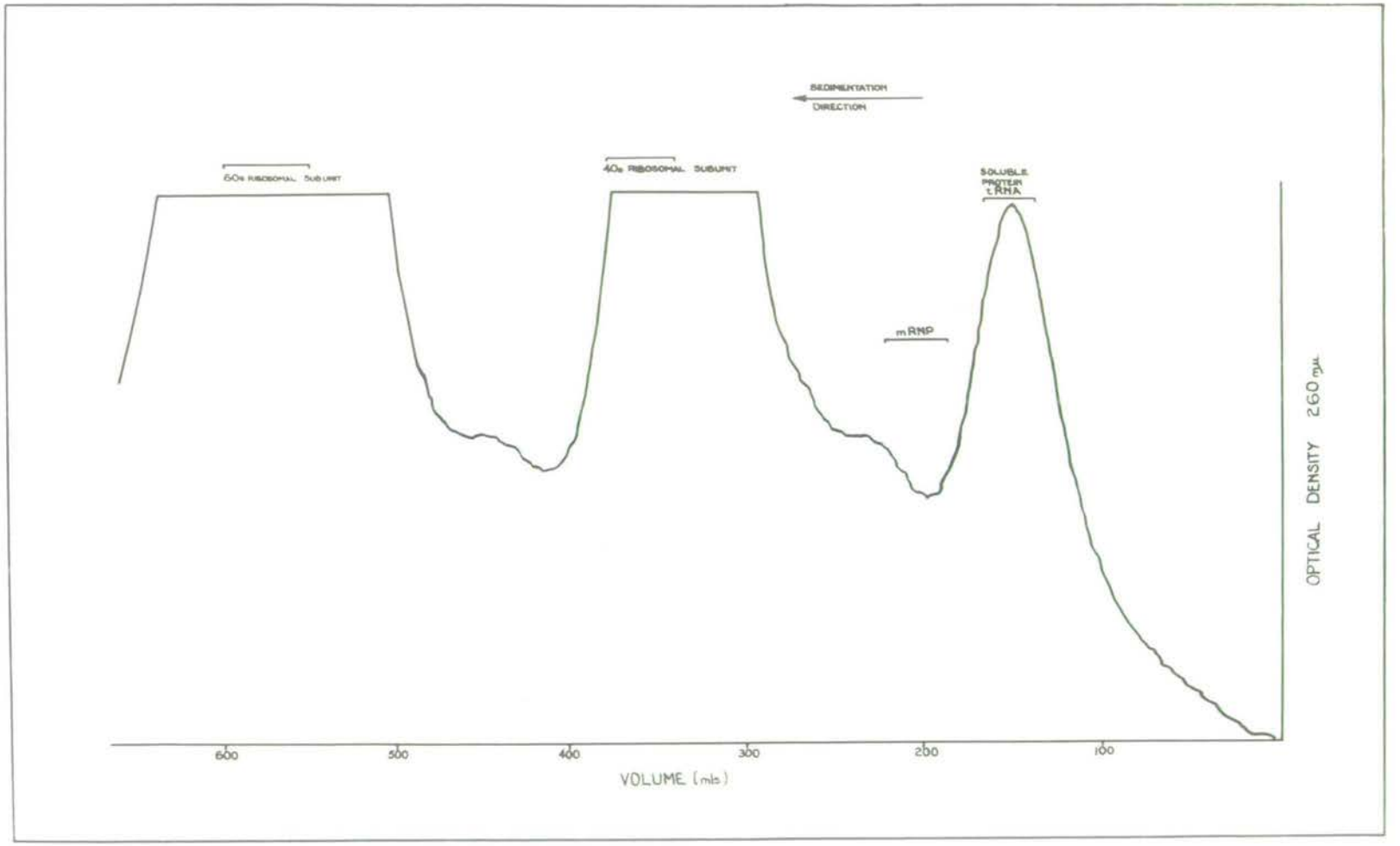


Figure 39. Zonal ultracentrifugation of EDTA-treated chick lens polysomes. Chick lens polysomes were washed in 0.5 M KCL and dissociated in 33 mM EDTA. 360 A_{260} units of high salt washed polysomes were then fractionated on a 15-40% (w/v) convex exponential sucrose gradient. Centrifugation was at 40,000 rev./min. for 17 hours, all other conditions as in Figure 36.



mRNA species were associated with the same RNA-binding proteins. This enhanced resolution was sought by using increased centrifugation times.

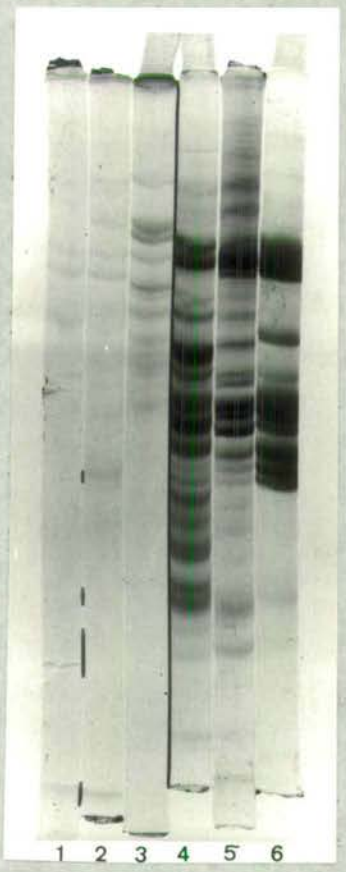
The sedimentation pattern of a second preparation of EDTA-treated polyribosomes through an exponential sucrose gradient prepared in a zonal rotor is shown in Figure (39). Under the centrifugation conditions specified the mRNP region was obtained again as a single broad zone. The region of the mRNP fraction was taken in order to minimise possible contamination of the particle by proteins stripped from the ribosomal sub-units by the action of the EDTA. The fractions in the diagram were then compared by electrophoresis in SDS polyacrylamide gels (Figure 40). Since some of the gels stained very faintly a schematic representation of the electrophoretic pattern of the proteins taken from the various zonal fractions is shown to facilitate comparison (Figure 41).

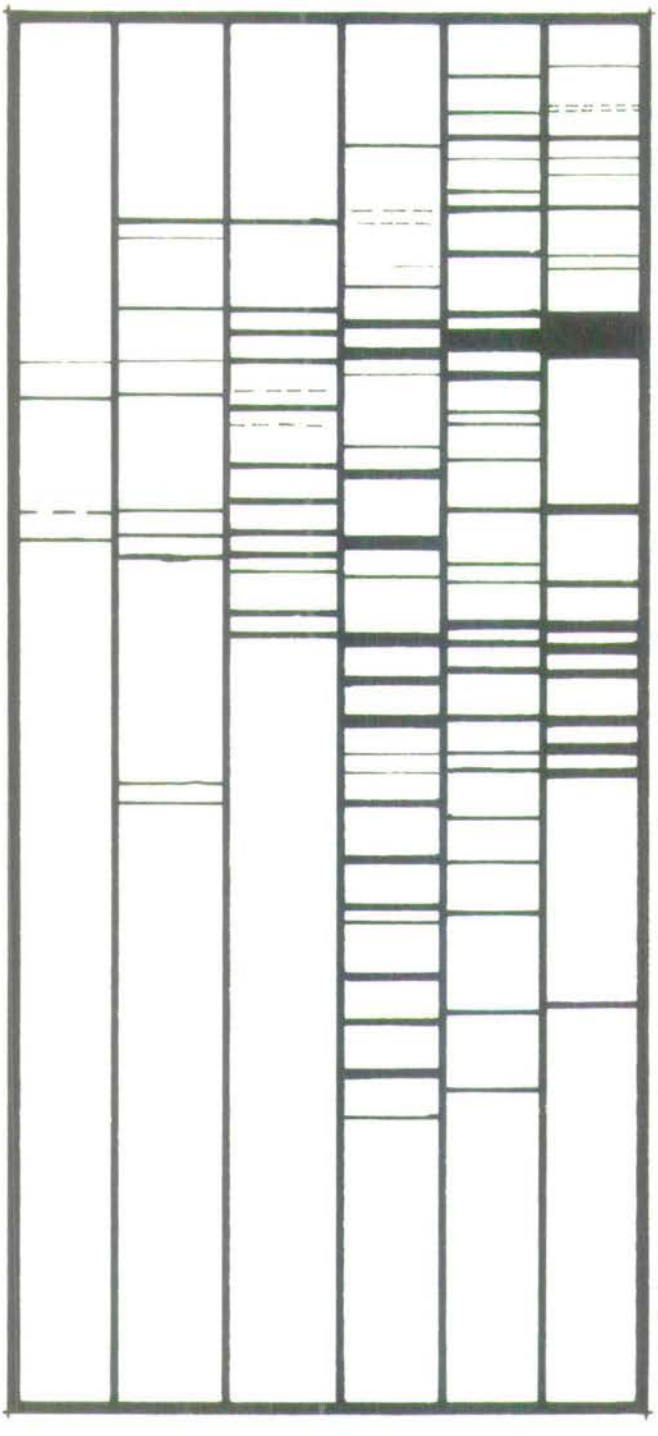
The three major high molecular weight components were again detected in the mRNP region together with 4 proteins of identical mobility to those found in the '4s' fraction, containing 4s RNA, 5s RNA and supernatant proteins. The three high molecular weight proteins appeared to be represented also in the small ribosomal sub-unit fraction.

In addition, comparison of the 0.5 M KCl "salt wash" of the chick lens polyribosomes with chick lens crystallins on SDS-polyacrylamide gels revealed that strongly staining bands in the "salt wash" showed identical electrophoretic mobilities to all the major components of the crystallin sample. Clearly the salt wash was removing many of the crystallins absorbed onto the ribosomes during the initial isolation procedure in low ionic strength buffers, together with a large number of other cytoplasmic proteins.

Figure 40 Polyacrylamide gel electrophoresis in the presence of SDS of the proteins associated with various zonal fractions. The samples were

1) 0.75 A_{260} units '4s' fraction
2) 1.5 A_{260} units mRNP fraction
3) 0.5 A_{260} units salt washed small ribosomal sub-units
4) 2 A_{260} units salt washed large ribosomal sub-units
5) 50 µg salt wash from polysomes
6) 50 µg chick lens crystallins.





1 2 3 4 5 6

Figure 41. Schematic representation of the electrophoretic pattern of the proteins taken from the various zonal fractions as shown in the previous figure. The samples were

1) 0.75 $O.D_{260}$ '4s' fraction
2) 1.5 $O.D_{260}$ fraction mRNP
3) 0.5 $O.D_{260}$ salt washed small ribosomal sub-unit
4) 2 $O.D_{260}$ salt washed large ribosomal sub-unit
5) 50 µg salt wash from polysomes
6) 50 µg chick lens crystallins.

Thus two separate zonal centrifugations of high salt washed polysomes from chick lenses had revealed that a minimum of three high molecular weight proteins appeared to be tightly bound to the mRNP particle liberated by EDTA-treatment. The problems remained as to whether different size classes of mRNA associated with an identical set of proteins, or were complexed in different proportions, or was each discrete mRNA species associated with only one of the tightly bound proteins? These types of question could be answered only by achieving increased separation within the mRNP region and analysing the protein components associated with different sizes of mRNA.

Increased resolution in the mRNP region was finally achieved by using a centrifugal speed of 43,000 rev./min for 17.5 hours (Figure 42). Under these conditions the mRNP fraction could be resolved into three discrete fractions together with a fourth fraction just separated from the small ribosomal sub-unit. Each of the indicated fractions were divided into two aliquots, one of which was subsequently processed for RNA electrophoresis, the other utilised for the analysis of the associated protein moieties. The gel electrophoresis pattern of the RNA isolated from the various fractions is shown in Figure 43. In addition, the electrophoretic conditions in 12 x 0.7 cm gels were chosen so that 18s RNA was just entering the gel, in order to estimate accurately the size of the various experimental fractions (Figure 44). Although in all cases the RNA was heterodisperse there was a distinct increase in the size of the RNA as the size of the RNP fractions increased, suggesting that the larger mRNP fractions were not merely artefactual aggregates of smaller mRNP particles. Table (13a) gives the estimated size of the various RNA samples.

The electrophoretic pattern of the proteins from the various fractions is shown in Figure (45). The analysis revealed two main

Table 13A Estimation of size of chick lens RNA samples from EDTA-treated ribmucleoproteins, from data shown in figs.43,44

Fraction	size of RNA from mRNP
a	5-10s, peak 9s.
b	peaks 12s, 14s.
c	peak 15s.
d	15-21s, peaks 16-17s, 18s.

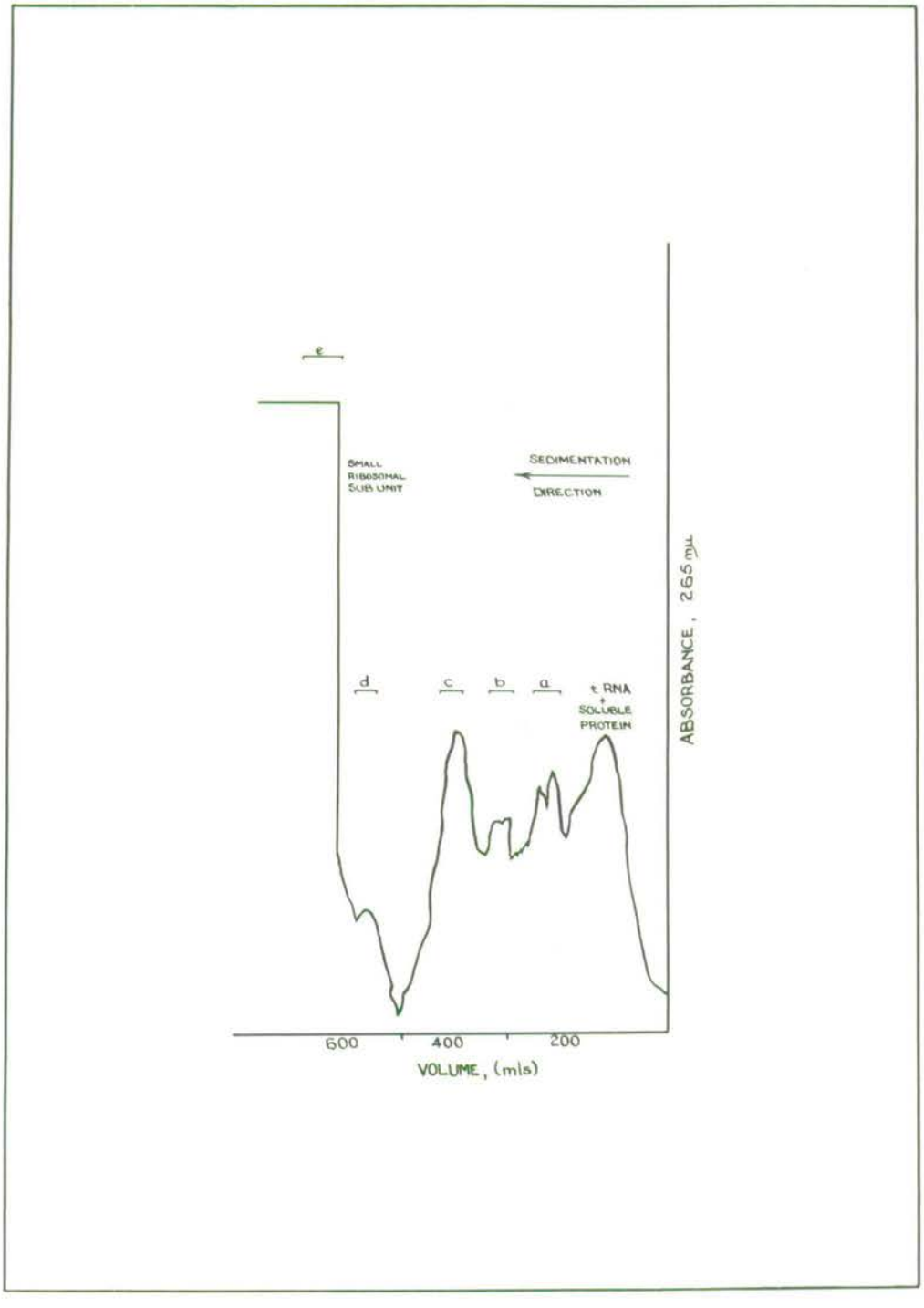
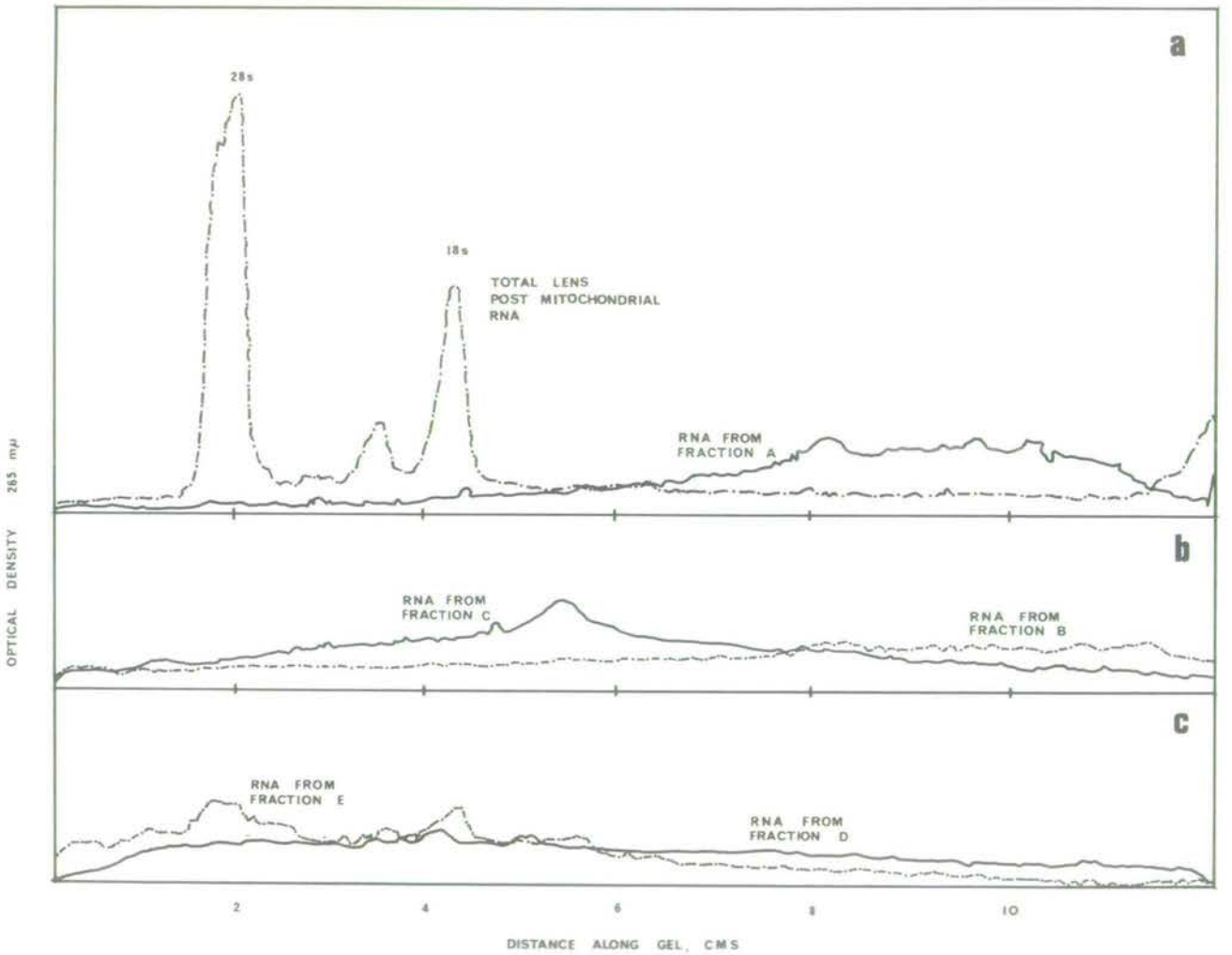


Figure 42. Zonal ultracentrifugation of EDTA-treated chick lens polysomes. Approximately 480 A_{260} units of high salt washed polysomes were dissociated in 33 mM EDTA and fractionated on a 15-40%(w/v) convex exponential sucrose gradient. Centrifugation was at 43,000 rev./min. for 17.5 hours. All other conditions as described in Figure 36.

Figure 43. 2.6% polyacrylamide electrophoresis of RNA samples isolated from EDTA-dissociated ribonucleoprotein fractions. Electrophoresis was performed, in gels 12 cm and 0.6 cm in diameter, at 5 mA/gel. All other conditions were as detailed in Figure 27. Loadings: total lens RNA 1 A_{260} unit (50 µg), zonal fractions 0.2 A_{260} units (10 µg). The amplification of the trace was obtained with the Servoscribe chart recorder set at 5v. deflection.

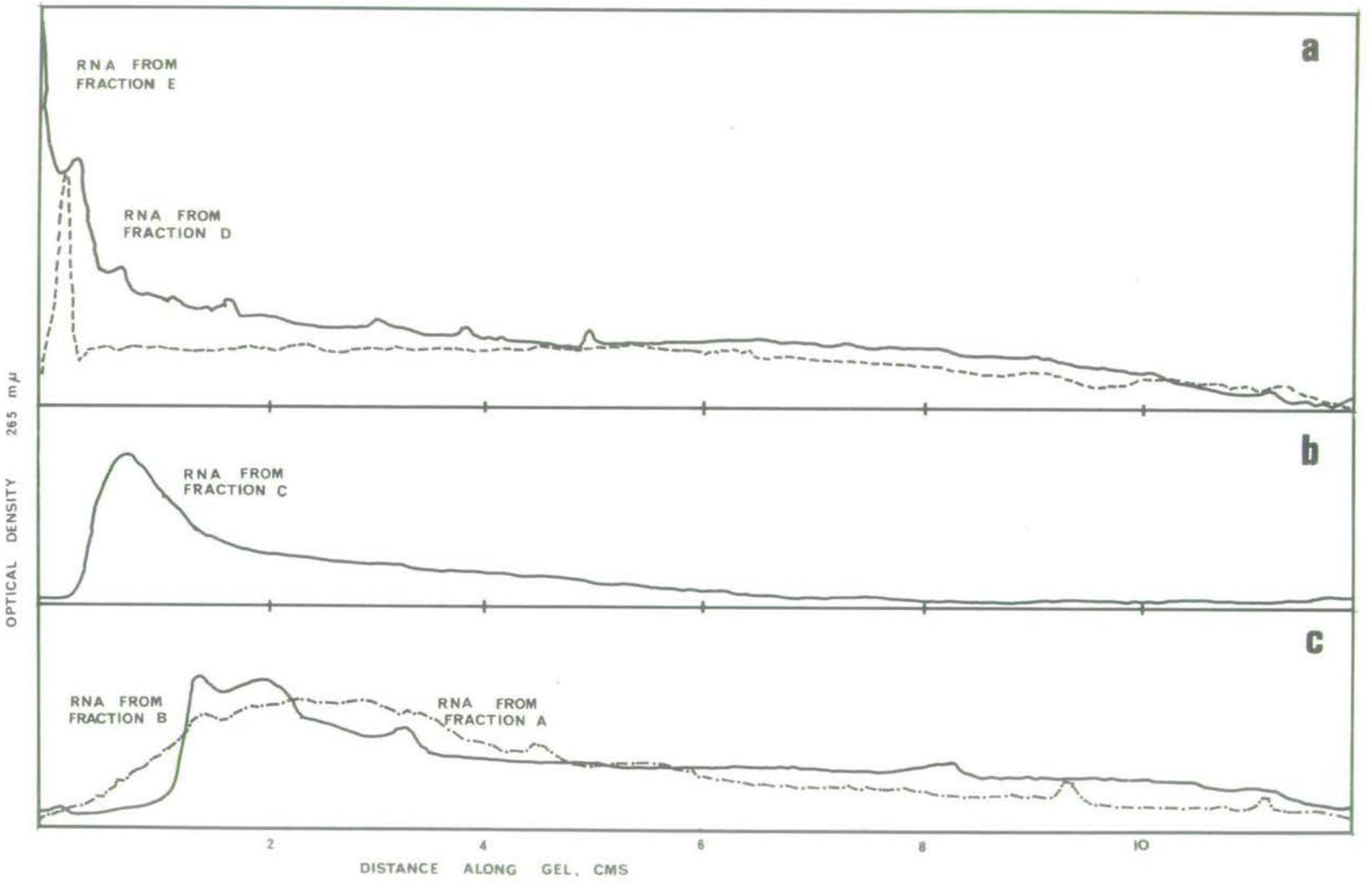


classes of protein: i) a group of 3 - 5 proteins, molecular weight 45,000 - 90,000, ii) a group of faintly staining proteins in the size range 35,000 - 20,000 daltons. A schematic representation of the electrophoretic pattern of these sets of proteins, together with that of a sample of lens protein is shown in Figure (46).

The lighter fractions sedimenting close to the supernatant fractions appeared obviously contaminated: indeed many of the bands showed identical electrophoretic mobilities to those of the major crystallin components. This smearing of the supernatant proteins may have been due to the increased centrifugation speed but was more probably due to incomplete salt washing in the preparation of the polysomes, leaving a considerable amount of cytoplasmic proteins to adsorb or co-sediment with the mRNP fractions. In addition the 3 polypeptides of high molecular weight (arrowed), common to all 5 fractions, clearly did not originate from crystallin contaminants. These three components were identical in electrophoretic behaviour to those identified as being tightly bound to RNA in the two previously described investigations.

There also appeared to be slight quantitative differences in the amount of each of the three components common to the different RNP fractions. The largest protein was most strongly represented in fractions a and b but appeared quantitatively similar to the other two components in fraction c. In addition although all three components appeared to decrease in quantity with increased size of the RNP fraction, the intermediate component appeared to be quantitatively less in fractions d and c. Obviously to clarify this point one would need to determine the molar ratios of protein to RNA within discrete RNP size classes. This would require in turn, the elimination of all protein contaminants and isolation of mRNA of widely different

Figure 44. 2.6% polyacrylamide electrophoresis of RNA samples isolated from EDTA-dissociated ribonucleoprotein fractions. Electrophoresis was performed in gels 12 cm long and 0.7 cm in diameter, at 2.5 mA/gel for 20 minutes followed by 5 mA/gel for 135 minutes. All other conditions were as specified in Figure 2. Loadings: fractions a, b and e 1 A_{260} unit (50 µg) and fraction d 0.5 A_{260} unit (25 µg). The amplification of the trace was obtained with the Servoscribe chart recorder set at 20 v deflection.



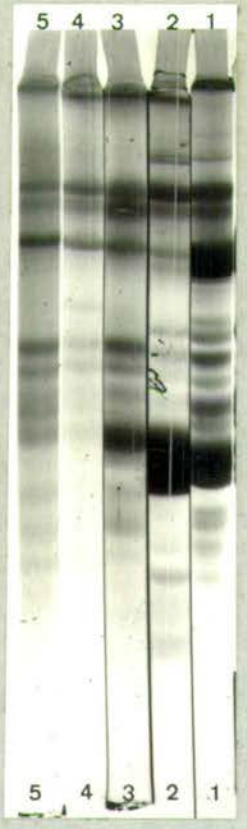


Figure 45 Urea - Acrylamide gel electrophoresis in 15% sodium dodecyl sulphate employing a discontinous buffer system. The origin is at the top and the samples are

1) 1 A_{260} unit fraction A,
2) 1 A_{260} unit fraction B,
3) 1 A_{260} unit fraction C,
4) 1 A_{260} unit fraction D,
5) 0.2 A_{260} unit fraction E, small ribosomal sub-unit.

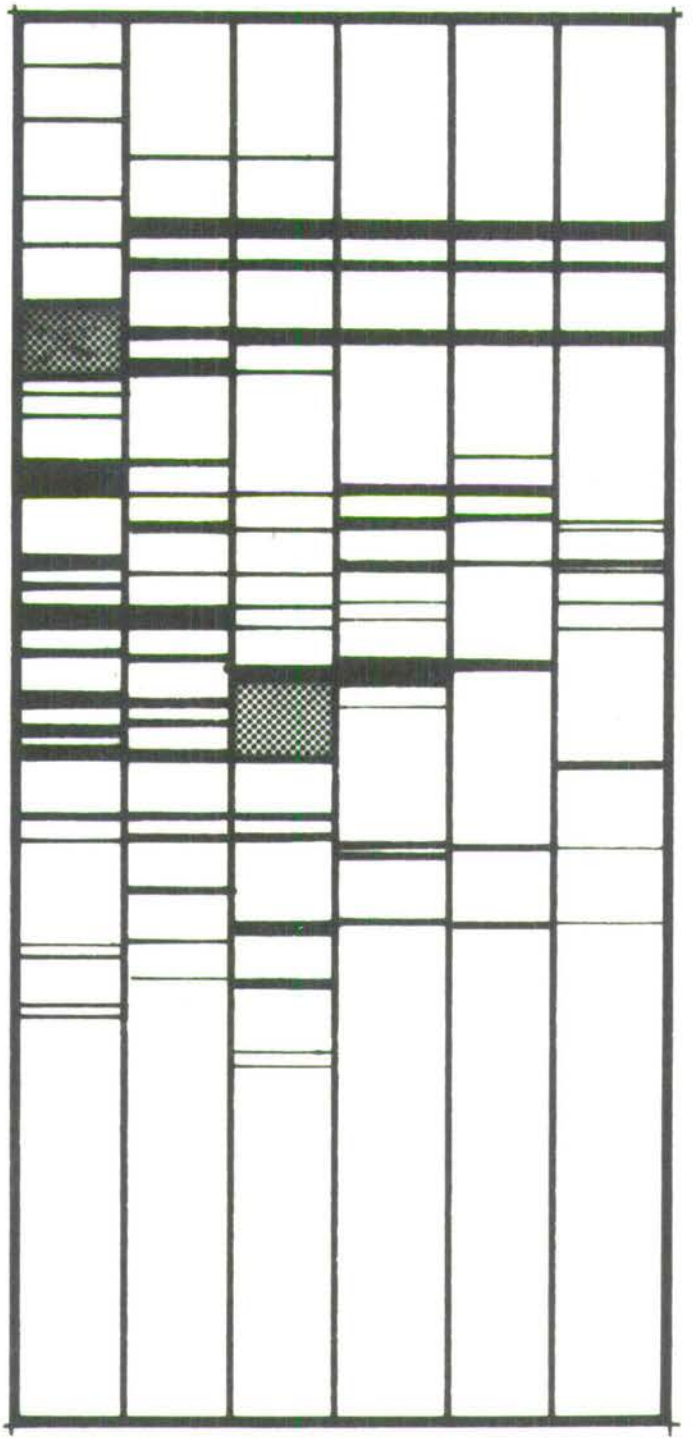
Samples were treated with 8 M urea, 0.1% beta-mercaptoethanol and 0.1% SDS before electrophoresis as described in the Materials and Methods section. The arrows indicate high molecular weight proteins common to all 5 fractions.

size classes. However the analysis described here suggested, at least, the possibility that different species of mRNA could be associated with the same tightly bound protein components but in quantitatively different proportions.

Thus three high molecular weight proteins were common to three separate preparations of mRNP fractions from EDTA-treated polysomes of chick lens. Although other proteins were associated with particular RNP fractions, only these three components were represented in all preparations. The electrophoretic mobilities of these components were calculated according to the method of Weber and Osborn (1969) and compared against a calibration curve constructed by running proteins of known molecular weights on urea-SDS polyacrylamide gels (figure 47). From this calibration curve the two major protein components were estimated to have molecular weights of 60,000 and 47,000 whilst the molecular weight of the intermediate component was estimated to be 56,000.

In summary, the analysis of the protein moiety of the mRNP particle released by EDTA dissociation of chick lens polyribosomes revealed that three high molecular weight proteins were associated with chick lens mRNA. When the mRNP was obtained as four separate size classes, three components of molecular weight 60,000, 56,000 and 47,000 were common to all fractions. In one particularly clean separation of the entire mRNP region, these three components formed the bulk of the protein associated with the mRNA, only three other very faintly staining components were present. There is, at least, an indication however that the different mRNA species within the mRNP fraction may be associated with quantitatively different amounts of these three major protein components.

The major conclusion reached here, that discrete polysomal mRNA species of the lens are associated with the same mRNA binding proteins, is examined, in the light of other authors' results, in the Discussion section (Chapter 15).



1 2 3 4 5 6

Figure 46. Schematic representation of the electrophoretic pattern shown in Figure 42 of the proteins obtained from the various zonal fractions together with a sample of chick lens crystallin. The samples are

1) 200 µg chick lens crystallins
2) 1 $O.D_{260}$ fraction a (tube Nos.21-28)
3) 1 $O.D_{260}$ fraction b (tube nos.29-34)
4) 1 $O.D_{260}$ fraction c (tube Nos.38-44)
5) 1 $O.D_{260}$ fraction d (56-62)
6) $0.20.D_{260}$ fraction e (65-70)

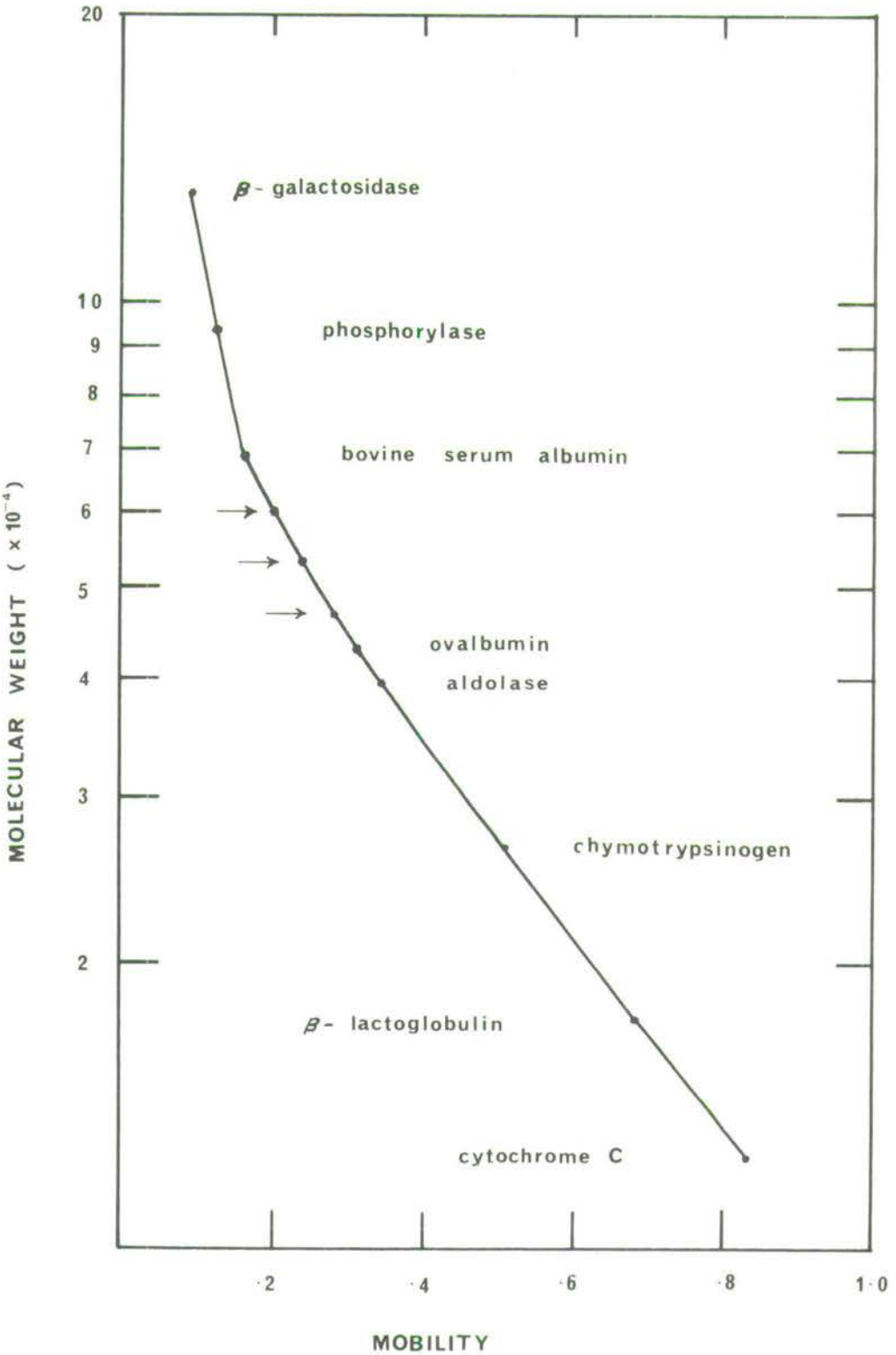


Figure 47. Estimation of the molecular weights of the proteins associated with chick lens mRNA. The protein markers employed in the determinations were run on individual gels in the amounts and conditions described in the Materials and Methods section. Mobilities were calculated according to the method of Weber and Osborn (1969). The arrows indicate the mobilities of the proteins associated with chick lens mRNA.

Chapter 12

Affinity Chromatography on Oligo (dT) - Cellulose of Dissociated Polysomes from Mouse Reticulocytes.

Lindberg and Sundquist (1974) showed that the components of EDTA-dissociated polysomes of KB cells could be fractionated by affinity chromatography on oligo (dT) - cellulose into an unadsorbed fraction containing more than 95% of the ribosomal sub-units and a fraction, retained by the column matrix, containing mRNA plus protein. This technique appeared to be a highly convenient method for isolating mRNPs containing poly (A) sequences. Consequently a systematic investigation of this technique was initiated, employing mouse reticulocyte cells, since globin mRNP particles are the most highly characterised of all such complexes. It was thus hoped to develop a suitable fractionation technique so that chick lens mRNPs isolated by this method could be compared to those obtained by zonal centrifugation of dissociated polysomes.

Preparation and chromatography of mouse reticulocyte polysomes.

The technique employed was, in essence, identical to that developed by Lindberg and Sundquist (1974) and is fully described in the Materials and Methods section. Preliminary investigations revealed a certain amount of RNA degradation was occurring during the fractionation procedure, so certain modifications were introduced. Polysomes were dissociated by puromylin treatment at 37°C (Blobel, 1972) in case nucleases were being released by the action of EDTA on mitochondrial membranes. All affinity chromatography was then carried out at 4°C to minimise nuclease activity. Finally, high salt washed polysomes, prepared according to the method of Blobel (1972) were used in order to minimise non-specific binding of cytoplasmic proteins, including nucleases, to mRNA. However it should be noted that in the cleanest reticulocyte preparations, the pattern of the major polypeptides of the adsorbed fractions, as revealed by electrophoresis in SDS -

polyacrylamide gels, were very similar, regardless of whether the polysomes were prepared in high or low salt conditions, or whether they were dissociated by EDTA or puromycin_7. The modifications, as judged by polyacrylamide electrophoresis of the RNA of the adsorbed fractions, did significantly reduce the degree of RNA degradation.

Variation in properties of oligo (dT) - cellulose

When commercial oligo (dT) - cellulose, from batches routinely utilised for mRNA isolation, was employed in preliminary experiments for the isolation of mRNP particles, it was clear that its binding properties varied from batch to batch (Table 13). In addition to the variation in amount of bound material that could be recovered by formamide elution, it was also clear that the size distribution of RNA and the degree of ribosomal contamination varied widely in different preparations.

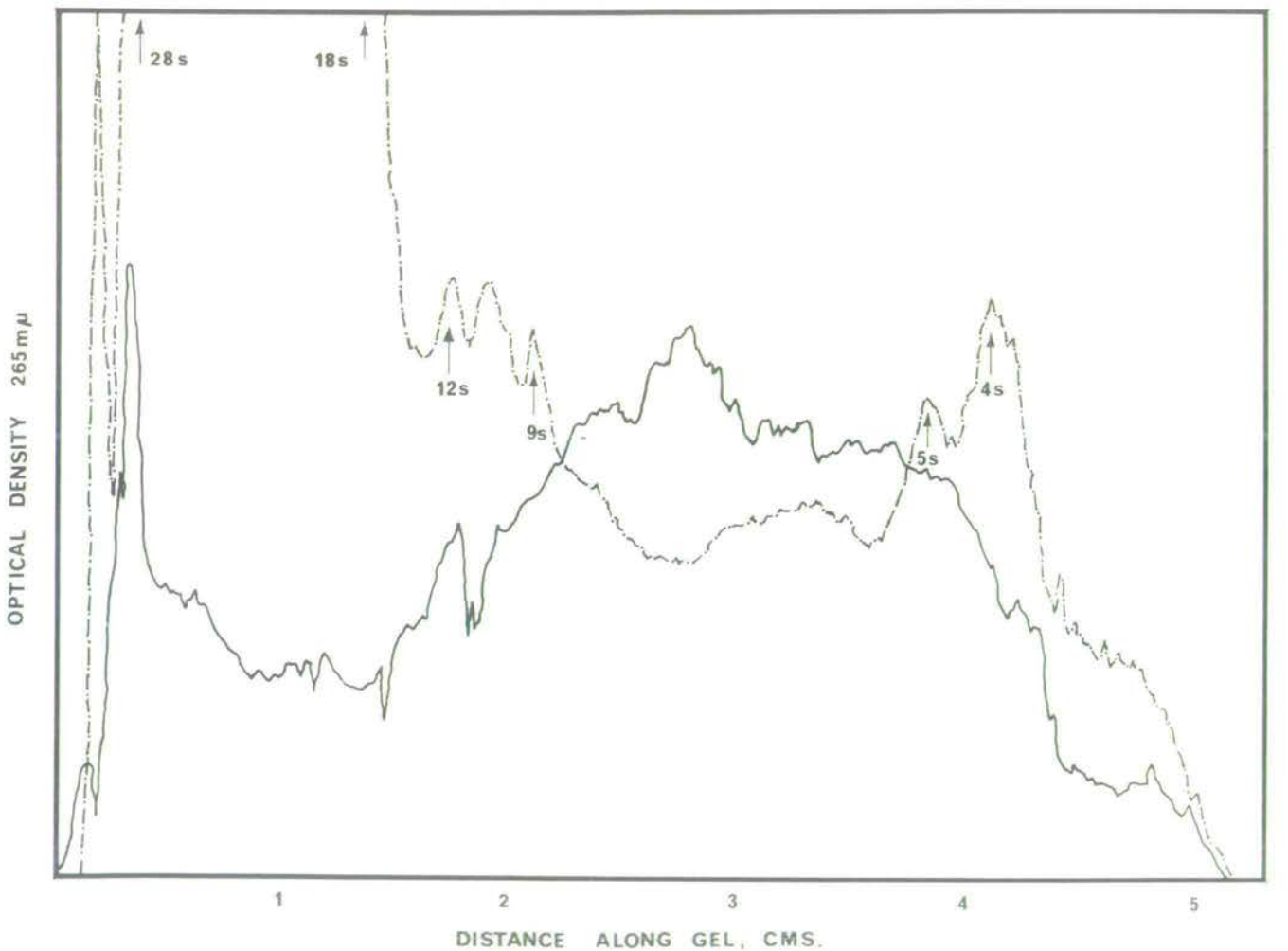
Lindberg and Sundquist (1974) noted that the oligo (dT) - cellulose preparations that would bind mRNP complexes reversibly were less effective in binding free mRNA. Consequently several batches of oligo (dT) - cellulose were obtained from G,D. Searle and Co. Ltd., (High Wycombe, Bucks., U.K.) which had been found during commercial testing to be less efficient than normal in binding free mRNA. These preparations were compared one immediately after the other, using the same sample of reticulocyte polysomes in all cases, to determine the degree of variation between batches and to obtain the most suitable preparation for efficient binding of mRNP complexes. The results of this comparison are set out in Table (14).

It can be seen again that there is considerable variability in the amount of bound material that can be recovered by formamide elution. Much of the bound material could only be released by .1 N

NaOH elution: presumably much of this fraction represents non-specifically bound material. The amount of material released by formamide varies from 20% to 80% of the total amount of material retained by the column (- final column of Table 14b). The most suitable column, as judged by polyacrylamide gel electrophoresis of the RNA components of the formamide eluates (data shown below) yielded only 50.9% of the total bound material upon formamide elution. Lindberg and Sundquist (1974) found that with commercial oligo (dT) - cellulose with a specified oligo (dT) chain length of up to 10 nucleotides, only 40% to 50% of bound material could be recovered by formamide elution. The degree of variability in RNA size distribution was much reduced with these oligo (dT) preparations suggesting they were more effedtive in binding mRNP complexes than those that bound free mRNA efficiently.

As these authors point out (Lindberg and Sundquist, 1974) the reason for variations in binding properties of oligo (dT) - cellulose are not known. However the aromatic components of lignin or lignin-like constituents of cellulose preparations have been implicated in the binding of polyadenylate to cellulose (DeLarco and Guroff, 1973; Sullivan and Roberts, 1973). Presumably similar types of binding sites in the cellulose matrix are responsible for the retention of oligo (dT) tracts and clearly this type of compositional feature can vary from preparation to preparation. It has been recently reported that the binding of poly (A) to cellulose is strongly influenced by the nature and concentration of the principal monovalent cations of the chromatographic solvent (Kitos et. al. 1974). It is conceivable that the salt conditions used to promote binding of oligo-dT tracts to cellulose may not have been optimum in the commercial techniques.

Figure 48. Gel electrophoresis of RNA in the 25% formamide fraction obtained by affinity chromatography on oligo (dT)-cellulose of puromycin-dissociated polysomes of mouse reticulocytes. Electrophoretic conditions were as described in the Materials and Methods section and in the legend to figure 37. For comparison a sample of total mouse reticulocyte RNA was electrophoresed at the same time. 50 µg (1 O.D.) of total RNA were loaded and 8 µg (.16 O.D.) of the formamide eluate fraction. Gel traces are shown for 1v. scale of deflection on the servoscribe chart recorder.



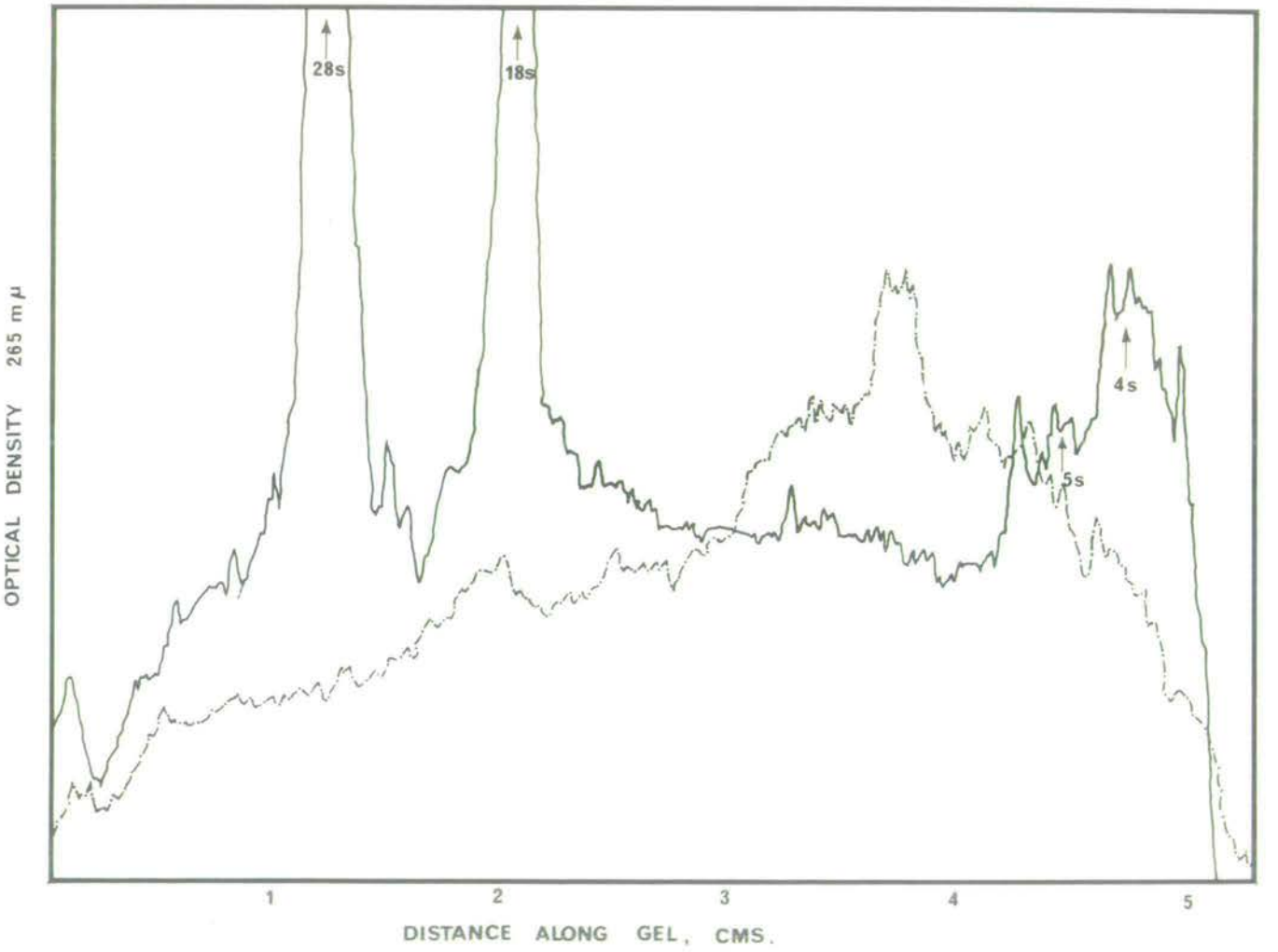
These considerations, on possible sources of variation in preparations, may be crucial to binding very short chain lengths of nucleotides to cellulose, for it is known that short tracts of poly (A) bind poorly compared to longer tracks of the homopolymers (Sullivan and Roberts, 1973).

The basis of preferential binding of mRNA complexed with proteins to oligo (dT) - cellulose is not known (Lindberg and Sundquist, 1974) nor is it likely to be understood until the type of interaction between the mRNP particle and cellulose is defined. Attempts to purify mRNP complexes by a second round of chromatography were unsuccessful. Samples eluted from the oligo (dT) - cellulose by 50% formamide were diluted by nine volumes of equilibration buffer and reapplied to the regenerated column. In no case did these samples in 5% formamide rebind to the oligo (dT) - cellulose. The physiological properties of the protein components may have been altered by the formamide. It is known that the proteins reassociate with the RNA when the formamide concentration is lowered (Lindberg and Sundquist, 1974). If however the action of formamide is analgous to that of formaldehyde in crosslinking the protein to RNA (Spirin et. al. 1966) then the requisite protein properties such as specific binding sites or conformational state, responsible for the interaction between the polypeptides and the adsorbent, may be destroyed.

Analysis of RNA size distribution of fractions from oligo (dT) - cellulose chromatography

RNA was analysed on 2.6% polyacrylamide gels, in tubes 7 cm long with 0.63 cm internal diameter, as described in the Material and Methods section. In general the size distribution in the formamide eluates varied widely. In some cases the fractions clearly consisted of mainly 28s and 18s ribosomal RNA. However even where ribosomal

Figure 49. Gel electrophoresis of RNA in the 25% formamide fraction obtained by affinity chromatography on oligo (dT)-cellulose of EDTA-dissociated polysomes of mouse reticulocytes. Electrophoretic conditions were described in the legen to fig. 37. 25 µg (.5 OD) of total RNA were loaded and 8 µg (.16 OD) of the formamide-eluted fraction. Gel traces are shown for 1v. scale of deflection on the servoscribe chart recorder.



contamination was obvious, some enrichment of material in the expected messenger range of 12s to 7s was usually obtained. A typical size distribution of the RNA in a formamide fraction obtained by affinity chromatography of puromycin-dissociated polysomes of mouse reticulocytes is shown in Figure 48, together with a sample of total RNA from mouse reticulocyte for comparison. It can be clearly seen that although there is some obvious contamination by 28s ribosomal RNA, the bulk of the RNA ranges in size from about 13s to 4s, with a major peak at about 8 - 7s. Even less obvious ribosomal contamination is shown in Figure 49, where the formamide fraction was obtained by affinity chromatography on oligo (dT) - cellulose of EDTA-dissociated polysomes of mouse reticulocytes. Again the bulk of the material is found in the size range characteristic of reticulocyte mRNA.

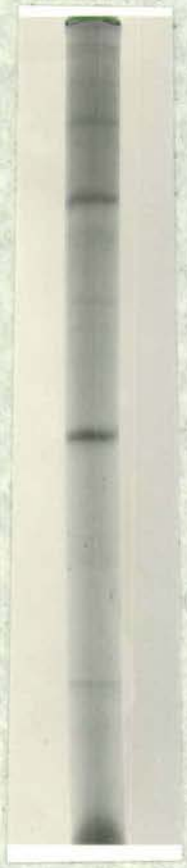
The results of Lindberg and Sundquist (1974) indicated that a certain amount of ribosomal contamination was to be expected with this technique. The exact amount of contamination is difficult to evaluate from their results on KB cells since the size distribution of the RNA in their formamide fractions falls mainly in the 30s to 18s range. This coincidence of messenger size with ribosomal RNA size does not of course occur in mouse reticulocytes, where the size of the globin mRNA is well characterised at 9s (Williamson et al., 1971) Therefore it was very encouraging to find that the bulk of the adsorbed fractions was of the requisite size for mRNA, from both puromycin and EDTA-dissociated polysomes. Since this size distribution could be obtained by partial degradation of ribosomal RNA, ultimate proof that these fractions are made up, in the main, by mRNP species will require their translation in an in vitro cell free system. These results however, taken together with the findings of Lindberg and Sundquist (1974) that their RNA patterns from the adsorbed fractions

of KB cell polysomes closely resembled the patterns obtained with polysomal mRNA isolated by poly(U)-sepharose, suggest strongly, but do not prove, that the bulk of the polysomal material retained and eluted from the column of oligo (dT) - cellulose is made up of mRNA species.

Analysis of polypeptide patterns of fractions from oligo (dT) - cellulose chromatography of mouse reticulocytes.

Although the size distribution of RNA in adsorbed fractions varied considerably from column to column, as did the degree of ribosomal RNA contamination, the polypeptide patterns of such fractions were much more consistent. The result of the polypeptide analysis by SDS polyacrylamide electrophoresis of a 25% formamide eluate obtained by affinity chromatography of EDTA-dissociated polysomes of mouse reticulocytes is shown in Figure 50. The corresponding analysis of the size distribution of RNA contained in this fraction was shown in Figure 49. Similarly figure 51 shows the polypeptide analysis of the formamide fraction whose corresponding RNA size distribution was shown in Figure 48. Four main polypeptides were common to both fractions, bands of identical mobility to these were, in fact, routinely separated in most preparations, even where ribosomal contamination was shown to be extreme, by RNA electrophoresis. The fact that consistent and limited polypeptide patterns on SDS polyacrylamide gels could be obtained regardless of whether the polysomes were prepared initially in high or low salt conditions, or whether they were dissociated by EDTA or puromycin, testifies to the efficiency of the final ionic conditions (0.2 M NaCL) used in the affinity chromatography to minimise artefactual mRNA-protein interactions.

Figure 50. The polypeptide composition of the 25% formamide fraction from oligo (dT)-cellulose chromatography of EDTA-dissociated polysomes from mouse reticulocytes, as revealed by SDS-polyacrylamide gel electrophoresis. 110 x 4 mm cylindrical gels were electrophoresed for 2.5 hours in the conditions described in the Materials and Methods section. (The size distribution of the RNA from this fraction was shown in figure 49).



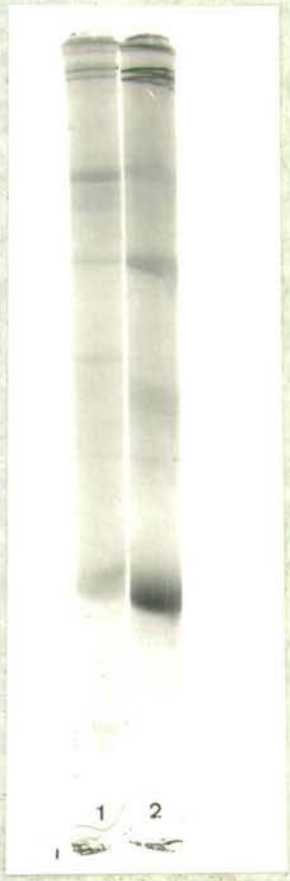
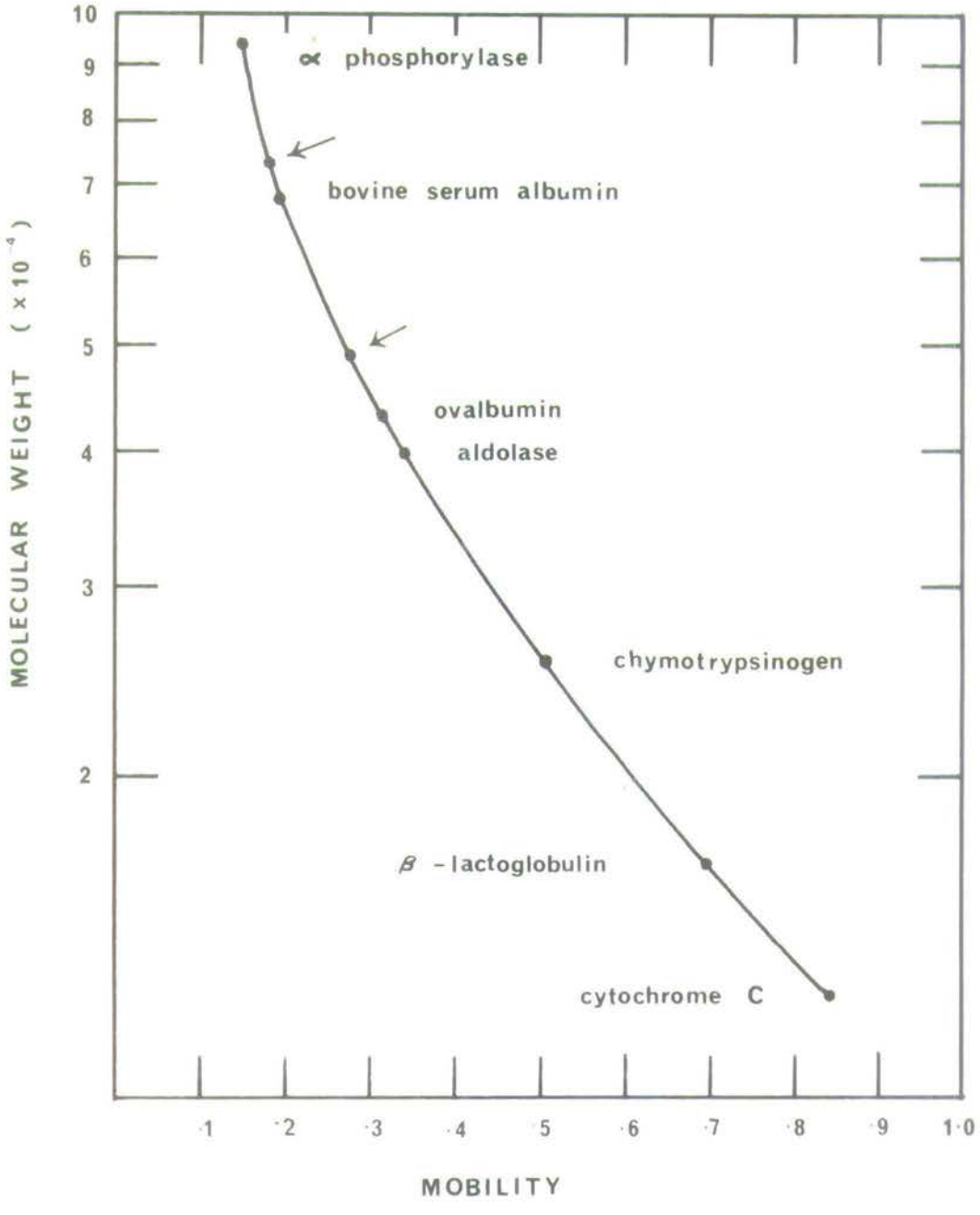


Figure 51. The polypeptide composition of 25% formamide fractions from oligo (dT)-cellulose chromatography of puromycin dissociated polysomes from mouse reticulocytes, as revealed by SDS-polyacrylamide gel electrophoresis. Experimental details as described in the legend to figure 50. Gel 1 - polypeptides fractionated by column 8 (Table 14). The size distribution of the RNA from this fraction was shown in figure 48. Gel 2 - polypeptides fractionated on column 9 (Table 14).

Figure 52. Estimation of the molecular weights of the two major polypeptides found in the formamide fractions obtained by oligo (dT)-cellulose chromatography of mouse reticulocyte polysomes. The protein markers employed in the determinations were run on individual gels in the amounts and conditions described in the Materials and Methods section. Mobilities were calculated according to the method of Weber and Osborn (1969). The arrows indicate the mobilities of the specified proteins.



The estimation of the molecular weights of the two major polypeptides found in the formamide fractions is shown in Figure 52. From these results their molecular weights appeared to be 73,000 and 49,000. Two other polypeptides (of 0.03 and 0.04 mobility, respectively) only just entered the gel. Because of the lack of marker proteins for this region only a minimum molecular weight of 140,000 can be estimated for these polypeptides.

The molecular weights of the two major proteins are in excellent agreement with the values obtained by Morel et. al. (1971, 1973) for duck globin mRNP proteins (73,000 and 49,000) and those for rabbit globin mRNP, as recently determined by Ernst and Arnstein (1975) who found values of 72,000 and 49,000. Blobel (1972, 1973) also reported values of 78,000 and 52,000 for rabbit globin mRNP and it can be seen from the steepness of the slope of the graph in these regions (Figure 52) that discrepancies in molecular weight estimations can derive from very minor experimental errors. These similarities in molecular weight are not restricted to reticulocyte cells, Lindberg and Sundquist found three major proteins of molecular weight 78,000, 68,000 and 56,000 in mRNP fractions of KB cells. Cerebral mRNP proteins from chick embryos were estimated to have molecular weights of 48,400 and 78,500 (Bryan and Hayashi, 1973). These authors also estimated the molecular weights of rabbit globin mRNPs to be 47,000 and 77,500. Similar proteins capable of binding mRNAs may have evolved in eukaryote species or alternatively the structure of mRNAs may restrict the range of protein sizes compatible to binding with various nucleotide configurations or sequences.

The polypeptides of very high molecular weight (140,000) may also be genuine components of mouse globin mRNP. Duck globin mRNP contains

minor components of 105,000 and 120,000 molecular weight. (Gander et. al. 1973) whilst mRNP proteins from KB cells contain two minor polypeptides of about 125,000 molecular weight (Lindberg and Sundquist, 1974). All the estimations of molecular weight were obtained by a SDS-polyacrylamide electrophoresis system similar to the one employed in this study.

Thus it was highly encouraging to discover that the sizes of the major polypeptides prepared by this chromatographic technique were in good agreement with those determined for reticulocyte globin mRNPs, isolated by centrifugation techniques. These results increase the possibility that intact reticulocyte mRNPs can be isolated by this new technique. However it must be borne in mind that proteins capable of binding poly (A)-containing mRNA might bind independently of the mRNA to the oligo (dT) stretches.

The consistency of the protein pattern obtained from analysis to analysis, in contrast to the variation in size distribution of the RNA in fractions retained by the column, suggests that the interaction between the mRNP complexes and the adsorbent may be mediated directly by the properties of the polypeptides rather than solely by the poly(A) segments of the mRNA. In the particular ionic conditions used it appears that non-specific binding of polypeptides to the adsorbent is much less marked than non-specific binding of ribsomal RNA. Lindberg and Sundquist (1974) presented a two-stage scheme in which the mRNA-associated proteins cause the retention of the complexes at low salt concentrations, the oligo (dT)-cellulose acting effectively as an ion-exchange column. The protein-column interaction can then be broken down by increasing the salt concentration, under which ionic conditions the main interaction is

through a stable poly (A)-oligo (dT) binding, subsequently broken down by the formamide.

If the mRNA-protein interaction is to some extent reversible, then the proteins may be free to interact with the oligo (dT) tracts. This independence of the binding properties of the polypeptides, based upon reversible dissociation could explain the consistency of the protein pattern. The data of Lindberg and Sundquist (1974) yield two pertinent pieces of information. Firstly whilst no quantitative differences in the RNA patterns were obtained as the concentration of the formamide buffer increased, the polypeptide pattern in the fraction eluted by 25% formamide was different from that of the fraction eluted by 50% formamide. The fraction eluted by 50% formamide showed only one major polypeptide of 78,000 molecular weight, whilst the fraction corresponding to the 25% formamide eluate contained polypeptides of 125,000, 78,000, 68,000 and 56,000. Clearly there are differences in tenacity of binding to the column amongst the different polypeptides. The finding that the binding of several of the polypeptides is dependent on formamide concentration, whilst the size distribution of the RNA in these eluates does not vary, indicates that a certain amount of dissociation can occur between the mRNA-protein complexes. The authors also presented isopycnic banding evidence that suggested that under the conditions of formamide elution from oligo (dT)-cellulose parts of the protein moiety may be lost from the mRNA-protein complexes. Re-association can occur at lower formamide concentrations.

Evidence was obtained in one investigation of the polypeptide patterns of formamide fractions that the four main polypeptides of high molecular weight, routinely detected in all preparations, differed in binding properties. As shown in figure (53), the fraction

eluted with 25% formamide contains only one major polypeptide (molecular weight 73,000), (see fig.48) whilst the 50% formamide eluate contains, in addition, two strongly staining proteins (molecular weight approximately 140,000) as well as several ribosomal contaminants. The fourth of the commonly found polypeptides (molecular weight 49,000) was only eluted, in this case by 90% formamide. This result was exceptional, in that generally the 25% or 50% formamide fractions contained all four polypeptides of these molecular weights. It does however, indicate that, at least on certain oligo (dT)-cellulose columns, the mRNA-protein complexes may be bound with quite different degrees of tenacity. Here the polypeptide of 49,000 molecular weight appears to be the most tenaciously bound, in contract to the finding of Lindberg and Sundquist, (1974) with KB cell preparations, that a polypeptide of 78,000 molecular weight was most strongly bound.

These apparent differences in binding properties of the polypeptides with the adsorbent might reflect different degrees of binding and dissociation between the mRNA and the polypeptides. It will be of extreme interest if these differences in binding properties are confirmed, since Scherrer and his colleagues have put forward a hypothesis to explain differential messenger stability that relies on different dissociation constants between mRNA molecules and their associated proteins (see Introduction, section II).

Summary

Affinity chromatography by oligo (dT)-cellulose appears to be a promising method for the isolation of mRNPs from mammalian cells. The major reservations expressed here are that a variable degree of contamination by ribosomal RNA can occur and absolute proof must be offered that the bulk of the low molecular weight RNA is composed

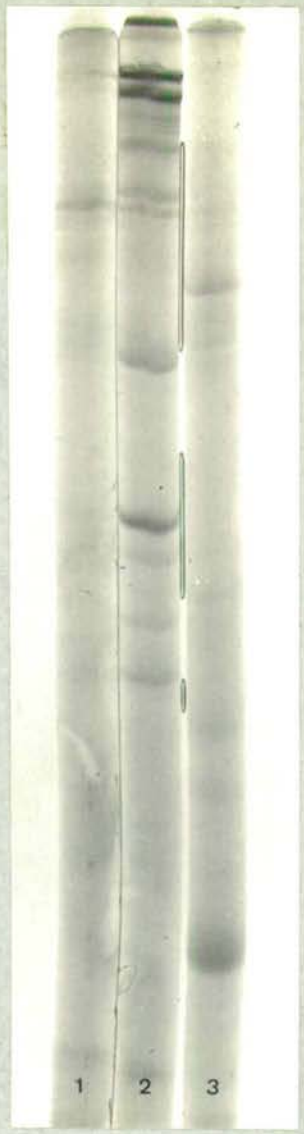


Figure 53. The polypeptide composition of fractions eluted by various concentrations of formamide from oligo(dT)-cellulose chromatography of puromycin-dissociated polysomes from mouse reticulocytes. 576 ODs of polysomes were applied to the column and fractions eluted successively with 25%, 50% and 90% formamide. Each fraction was then analysed on 15% SDS-polyacrylamide gels as described in the Materials and Methods section. Gel 1 shows the 25% formamide eluate, gel 2 the 50% formamide eluate and gel 3 the 90% formamide eluate.

of mRNA species. At the least, however, it seems an efficient and conveniently quick method for separating major proteins of identical molecular weight to others shown to be mRNA-associated proteins by conventional techniques.

Table 13

oligo (dT) Batch/Column	O.D. Salt Washed Polysomes Applied To Column	% Total O.D. Eluted By Formamide Buffers
1	264	0.38
2	392	0.66
3	264	0.96
4	477	0.50
5	576	0.42

Comparison of the binding properties of several batches of commercial oligo (dT) - cellulose routinely used for mRNA isolation

Separate batches of high salt washed polysomes from mouse reticulocytes were dissociated with puromycin then applied to various oligo (dT) - cellulose columns, under the conditions described in the Materials and Methods section. The adsorbance at 260 nm was measured of all material displaced from the column by formamide buffers, after the usual salt-ethanol precipitation at -20°C.

Table 14a

oligo (dT) Batch/Column	O.D. Salt Washed Polysomes Applied To Column	% O.D. Eluted By 25% Formamide	% O.D. Eluted By 50% Formamide	% O.D. Eluted By . I N NaOH
6	100	0.17	0.05	0.88
7	150	0.39	0.07	0.39
8	150	0.22	0.08	0.29
9	150	0.27	0.12	0.10
10	200	0.12	0.08	0.44

Table 14b

Column	% Total O.D. Eluted by Formamide (a)	% Total O.D. Eluted by NaOH (b)	$\frac{a}{a+b}$
6	0.22	0.88	20%
7	0.46	0.39	54.1%
8	0.30	0.29	50.8%
9	0.39	0.10	79.6%
10	0.20	0.44	31.25%

Table 14

Comparison of the binding properties of several batches of commercial oligo (dT) - cellulose which bound free mRNA less efficiently than normal. A single preparation of high salt washed polysomes from mouse reticulocytes was dissociated ~~from mouse reticulocytes~~ then divided into aliquots. Each of the various oligo (dT) - cellulose columns was used for affinity chromatography of one aliquot, one immediately after another. Following elution with 25% or 50% formamide, non-specifically bound material was removed with . I N NaOH. The adsorbance at 260 nm was measured of all fractions after overnight salt-ethanol precipitation at -20°C. The data has been rearranged in table 14b to facilitate the comparison of the amount of material released by formamide to that initially retained by the column.

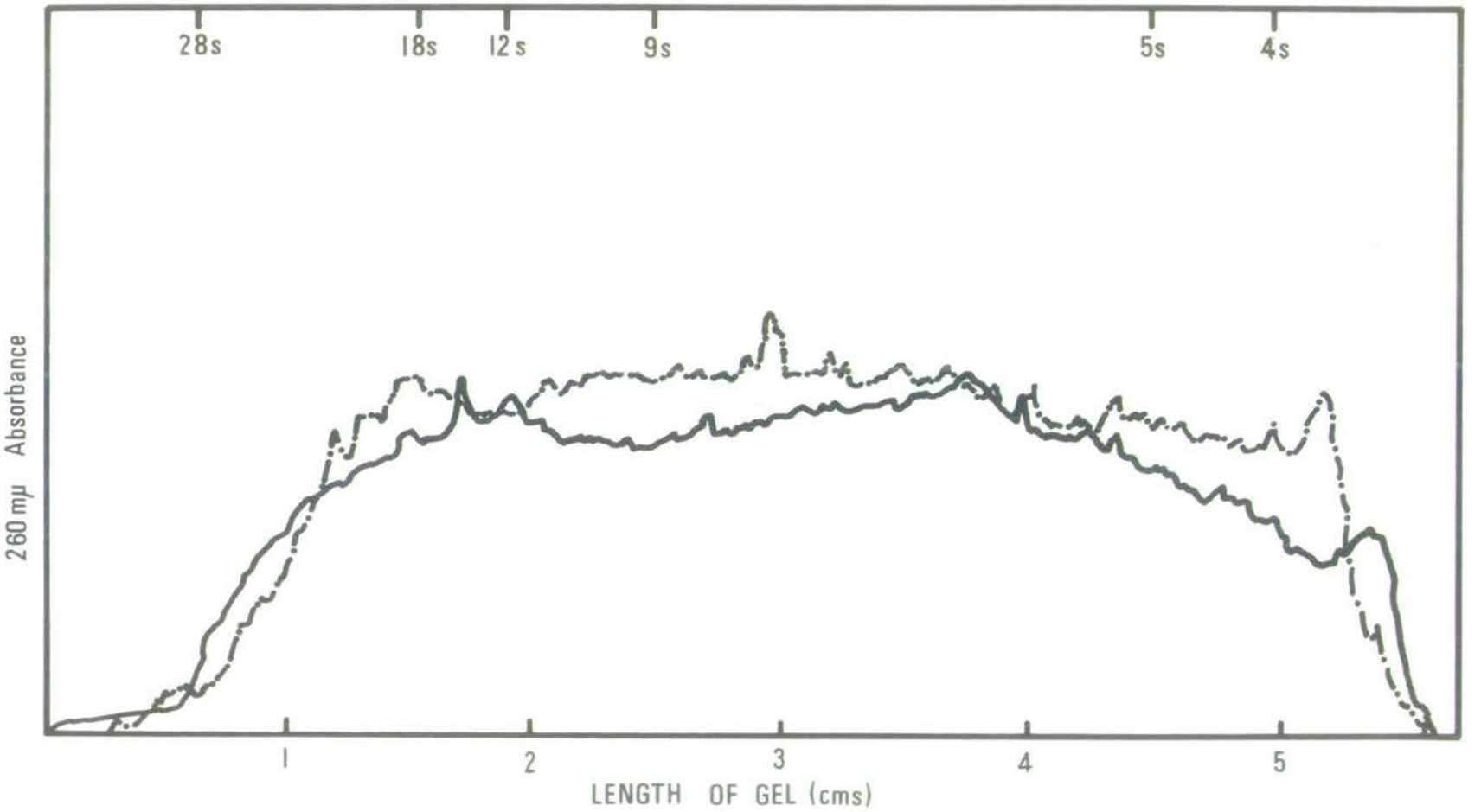
Chapter 13

COMPARISON OF THE POLYPEPTIDE COMPOSITION OF mRNP FRACTIONS FROM MOUSE RETICULOCYTES AND CHICK LENSES.

There is no reason to believe that the full complexity of mRNA-associated proteins would be necessarily revealed solely by SDS-electrophoresis, a technique that discerns only differences in molecular weight. Conceivably the configuration of mRNA molecules might impose strict stereochemical restrictions on such proteins, limiting them to a range of particular sizes capable of binding various nucleotide sequences or features of secondary structure. Such constraints might introduce a gross overall similarity in molecular weights of mRNA-associated proteins of different species, perhaps masking important differences in structure or properties. Consequently it appeared of value to compare the iso-electric points of these proteins from two different tissues, in order to determine whether major differences in charge existed between the two sets of proteins. The degree of binding of these proteins to mRNA molecules is likely to be influenced by the particular charge on a mRNA-associated protein.

As described in the Materials and Methods section, polysomes from 1 day post-hatch chicks were dissociated by puromycin and applied to the identical column of oligo-dT cellulose, that had previously been used to fractionate mouse reticulocyte polysomes (column 8 of table 14, fig.50) Each formamide fraction was divided into three aliquots, and precipitated in the described fashion with .4M salt and ethanol. One aliquot was utilised for RNA gel electrophoresis, the other two samples were used for polypeptide analysis: one sample fractionated by gel electrofocusing and the other submitted to SDS-polyacrylamide gel electrophoresis. These analyses were then compared to similar ones undertaken

Figure 54. Gel electrophoresis of RNA in the 90% formamide fraction obtained by affinity chromatography on oligo (dT)-cellulose of puromycin-dissociated polysomes of chick lenses. Electrophoretic conditions were as described in the Materials and Methods section and in the legend to figure 37. For comparison a sample of lens RNA prepared by chromatography on poly(U)-sepharose is also shown. Both samples were loaded at a concentration of about .1 O.D. (5 µg). Gel traces are shown for the 1v. scale of deflection on the servoscribe chart recorder. RNA size markers from a sample of total mouse reticulocyte RNA electrophoresed at the same time are also shown.



with samples from mouse reticulocytes.

Analysis of RNA size distributions of fractions from oligo (dT)-cellulose chromatography of chick lens samples

RNA was analysed exactly as described in the previous chapter. Results were in general similar to those found for mouse reticulocyte samples. Although ribosomal contamination was obvious in most cases, enrichment of material in the size range of 20s to 7s was usually obtained. A particularly clean separation of RNA in a 90% formamide fraction obtained by affinity chromatography of puromycin-dissociated polysomes of chick lenses is shown in fig. 54 together with a sample of RNA prepared by Dr. R. Williamson, of poly (A) containing RNA obtained by poly (U)-sepharose chromatography of a chick lens sample, according to published techniques (Williamson et al.1974). Similar fractions have shown delta crystallin template activity when translated in the wheat germ system (Burns, Williamson and Lanyon, unpublished results). The size distributions of these RNA samples are very similar, ranging in size from about 20s to 4s. Williamson et al.(1972) found that chick lens mRNA was hetero-disperse, ranging in size from 22s to 4s, with three peaks at 15s, 12, and 9s. Purified chick delta crystallin mRNA, isolated by oligo-dT chromatography, is approximately 20s in size (Zelenka and Piatigorsky,1974). Thus the results obtained were again encouraging in that the bulk of the absorbed fractions were of the requisite size for chick lens mRNA, as determined by polyacrylamide gel electrophoresis. However, as pointed out for the reticulocyte samples, proof that the adsorbed fractions are made up, in the main, by mRNP species will require their translation in an *in vitro* cell-free system.

Analysis of polypeptide patterns of fractions from oligo (dT)-cellulose chromatography of chick lens cell by SDS-polyacrylamide electrophoresis.

When the polypeptide patterns of formamide fractions obtained by oligo (dT)-cellulose chromatography of chick lens polysomes were analysed on 15% SDS-polyacrylamide gels, it was obvious by a comparison with a standard of total lens proteins from adult chicks, that many of the proteins from the adsorbed fractions migrated identically to the major crystallins (fig.55). Apparently contaminants remaining in the concentrated sample of high salt washed polysomes have adsorbed non-specifically to the column. This is not unreasonable considering the extraordinarily high protein concentration of the lens and the general adsorption problems experienced in routine chromatography of chick lens crystallins on media similar to cellulose (Truman 1968). In addition however four bands (arrowed) were present in the 50% formamide eluates from two separate preparations (gels 1 and 3, fig.55) that were not coincident with crystallin markers (gel 4, fig.55). Three of these bands were also found in the 90% formamide eluate (gel 2, fig.55).

The estimation of the molecular weights of these proteins is shown in fig.58 and compared in Table 15 to the molecular weights of the polypeptides previously isolated in chick lens mRNP fractions obtained by zonal centrifiguration. For comparison the results for mouse reticulocyte fractions, determined in the previous chapter, are also shown. The molecular weights of the three smallest polypeptides revealed by affirmity chromatography of chick lens samples are in excellent agreement to those found in

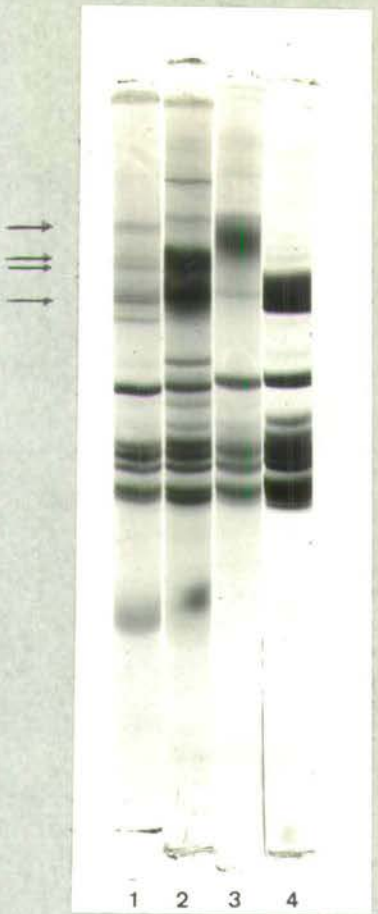


Figure 55. The polypeptide composition of various formamide fractions from oligo(dT)-cellulose chromatography of puromycin-dissociated polysomes from chick lenses, as revealed by SDS-polyacrylamide gel electrophoresis. Electrophoresis was performed as described in the Materials and Methods section. The samples are: 1) 50% formamide eluate and 2) the 90% formamide eluate of a single preparation of polysomes.

3)the 50% formamide eluate of a second preparation.

4) a sample of total lens protein from adult chicks (250µg).

zonal fractions of the same material. Polypeptides of higher molecular weight have occasionally been found in some, but not all zonal fractions (e.g. gels 1 and 2 of fig.45, chapter 11) and those may represent the polypeptides of 74,000 and 100,000 found in fractions separated on oligo (dT)-cellulose. It is however clear from the results reported in the previous chapter on mouse reticulocyte fractions, together with the findings of Lindberg and Sundquist (1974) that high molecular weight proteins may be revealed by oligo (dT) cellulose chromatography in addition to those found in mRNP complexes isolated by centrifiguration techniques. This additional complexity may be due to different ionic conditions in the two procedures. Alternatively these high molecular weight proteins may be easily detached from mRNA but bind to oligo (dT) stretches whereas in zonal separations they are centrifigured down with ribosomal proteins. However the correlation of size of the three smaller polypeptides with those determined for chick lens mRNP-proteins isolated by zonal centrifiguration was highly encouraging, indicating again that the chromatography technique could be used potentially to separate out mRNP complexes, provided non-specific adsorption of lens crystallins could be counteracted, presumably by rigorous salt-washing of the polysomes.

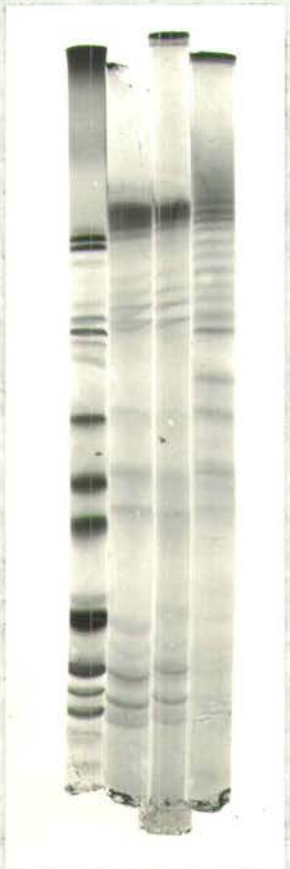
Mention was made in the previous chapter of the similarities in molecular weight of mRNP-proteins from many systems. It is striking that two of the polypeptides revealed by affinity chromatography of chick lens polysomes are very similar in molecular weight to the corresponding proteins of mouse reticulocyte cells (Table 15). It was of interest to determine whether these similarities extended to the overall charge of mRNP-proteins.

Comparison, by gel electrofocusing, of polypeptide patterns of fractions from oligo (dT)-cellulose chromatography of chick lenses and mouse reticulocytes.

When the iso-electric spectra of the polypeptides in the formamide eluates obtained from mouse reticulocytes and chick lens were compared, a very marked difference could be detected (fig.57) Again, disregarding any bands of iso-electric point coincident with those in a sample of total lens proteins from adult chicks, the remaining bands (arrowed) fall within a very restricted pH range of 4.0-5.0. In fact the number of bands assumed to be derived from mRNA-associated proteins may be underestimated, for the two major bands of lowest iso-electric point in the adult sample, which are found in this pH region, are not found in any great quantity in one-day post hatch chicks (see Chapter 14.) However regardless of the exact number of bands from mRNA-binding proteins revealed by this technique, their restricted pH range is entirely different from those polypeptides in the formamide eluates obtained from mouse reticulocytes. Contamination by cytoplasmic proteins is of much less account in the reticulocyte sample, given the comparative purity of the sample revealed by SDS-polyacrylamide analysis. However the iso-electric points of the polypeptides are again confined to a restricted pH range of 7.0-8.3, in complete contrast to those found for the polysomal sample from chick lens. Three major bands can be detected in the reticulocyte sample and 3-6 minor bands.

The final number of bands determined by this technique may be due to either genuine additional heterogeneity of polypeptides, revealed by the increased sensitivity of the gel electrofocusing

Figure 56. The polypeptide composition of various formamide fractions from oligo (dT)-cellulose chromatography of puromycin-dissociated polysomes from chick lenses, as revealed by gel electrofocusing in the presence of urea. Gel electrofocusing was performed as described in Chapter Three. The samples from left to right are: total lens protein from adult chick (250 µg), two samples from a 50% formamide eluate and a similar sample from a second preparation of polysomes.



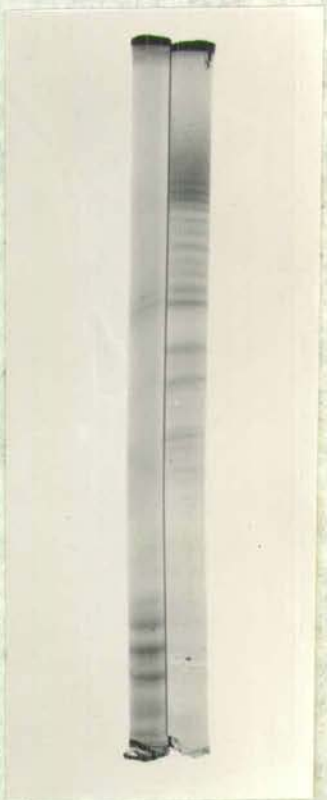


Figure 57. Comparison of the polypeptide composition of 50% formamide fractions from oligo (dT) chromatography of puromycin dissociated polysomes from mouse reticulocytes and chick lens, as revealed by gel electrofocusing. Gel electrofocusing was performed as described in Chapter Three. The reticulocyte sample (≃10µg) is on the left, the lens sample (≃12µg) on the right.

technique, or reflect a more limited number of polypeptides bound to differing lengths or amounts of RNA. Since the precipitates from the formamide eluates were dissolved in 8M urea prior to gel electrofocusing, i.e. in dissociating conditions, the former interpretation is favoured, thereby indicating that subtle differences in charge may exist between similarly sized polypeptides. This type of speculation could ultimately be resolved by treating similarly analysed fractions with specific RNA strains, to determine whether RNA is associated with the polypeptides.

However, it is clear from these results that mRNA-binding proteins from chick lens polysomes have much lower iso-electric points than those of similar proteins found in mouse reticulocyte polysomes, i.e. are more basic in overall charge than the reticulocyte proteins. This appears to be the first indication that major differences in charge may exist between two populations of mRNA-binding proteins. The work described in the previous chapter and the work of Lindberg and Sundquist (1974) indicated that differences in binding properties may occur within a set of such proteins of a single tissue. The demonstration that the range of iso-electric points for these proteins are restricted and highly different for two separate tissues also underlines the complexity of these proteins, which appears to be understated when only their molecular weights are examined. This complexity of charge favours schemes where the dissociation constants governing the interaction between the mRNA and proteins provide the basic mechanism determining the activity and stability of the mRNA (as suggested in Spohr et al.1970 and discussed in the Introduction). The significance of the findings described here is reviewed in the Discussion section (chapter 15).

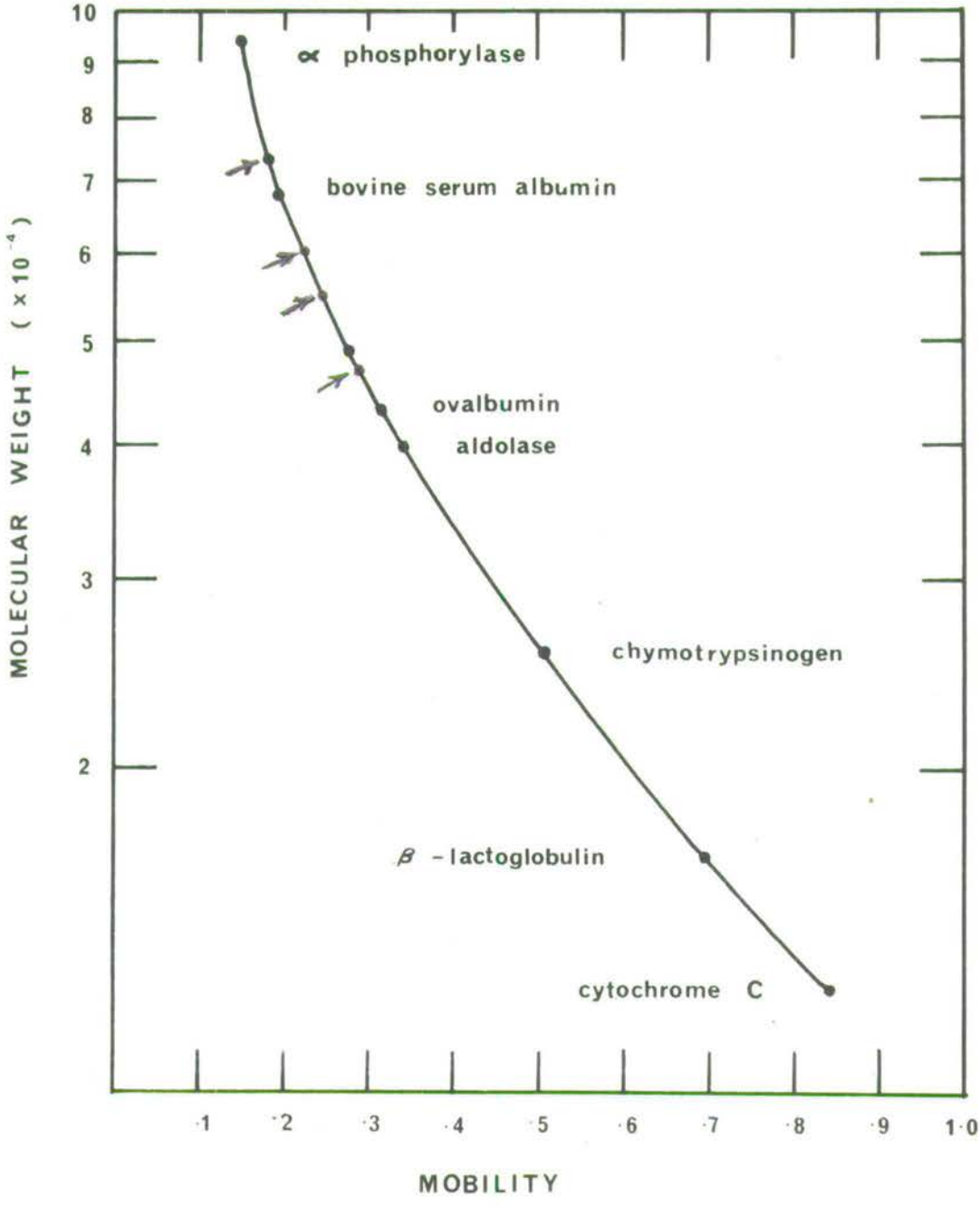


Figure 58. Estimation of the molecular weights of the major polypeptides found in the formamide fractions obtained by oligo (dT)-cellulose chromatography of chick lens polysomes. Calibration was performed in the same electrophoretic run previously described (fig. 52) for mouse reticulocytes. The arrows indicate the mobilities of the specified proteins.

Table 15

The molecular weights of the polypeptides associated with putative mRNP fractions from chick lens and mouse reticulocyte polysomes.

Preparation:	Chick Lens		Mouse reticulocyte
	Zonal Centrifugation	Affinity Chromatography	Affinity Chromatography
			2 polypeptides above 140000
		100,000	
		74,000	73,000
	60,000	60,000	
	56,000	56,000	
	47,000	48,000	49,000

CHAPTER 14.

EFFECT OF ACTINOMYCIN ON THE ONTOGENY OF THE POLYPEPTIDE CHAINS OF THE CRYSTALLINS DURING CHICK LENS DEVELOPMENT.

One constant aim of this work has been to investigate gene expression during differentiation by following the ontogeny of the individual polypeptides which make up the main structural proteins of the lens, the crystallins. After attribution of the origins of all of the major subunits was complete (see Chapter 8) an attempt was made to follow the ontogenic sequence of the polypeptide chains during chick lens development, as revealed by gel electrofocusing in dissociating conditions. Furthermore the resistance of synthesis of each polypeptide to actinomycin D treatment was followed using two radio-active isotopes of amino acids (see below). The experimental rationale thus combined two major points of interest throughout this entire investigation, namely, the subunit analysis of crystallins and the stability of the mRNAs coding for such polypeptides.

Urea polyacrylamide gel electrofocusing of lens proteins.

Under the conditions described in the Materials and Methods section, extracts of embryonic lenses were homogenised in 8M urea-100mM 2-mercapteothanol and electrofocused on polyacrylamide gels containing 6M urea (see Chapter 3). The results are shown in figs. (60 and 61) and summarised in fig. (62). Actual gel electrofocusing results are represented in this latter figure by unbroken lines. However allowance must be made for the variation in amount of protein applied, which depended on the availability of particular ages of embryos for dissection. In less concentrated samples, such as that of the 12-day embryo, only the most major of components can be detected, but the ontogeny of

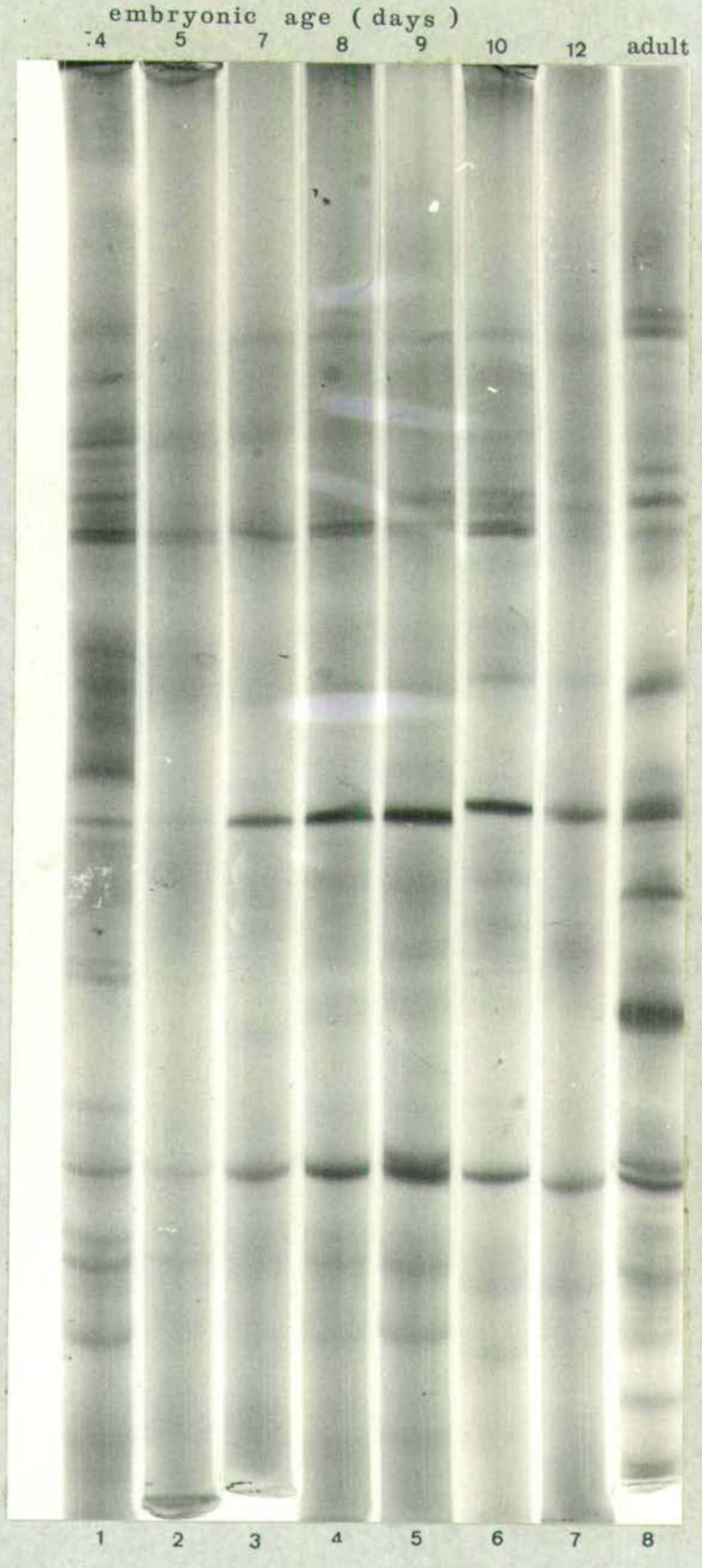


Figure 60a. Isoelectric focusing of early embryonic lens extracts on polyacrylamide gels of 6M urea. The groups represent embryonic ages analysed together in the same run. For comparison an adult sample of total chick crystallin (at 250µg) is also shown.

Figure 60b. Isoelectric focusing of early embryonic lens extracts on polyacrylamide gels of 6M urea. The groups represent embryonic ages analysed together in the same run. For comparison an adult sample of total chick crystallin (at 250µg) is also shown.

particular polypeptides can be deduced from more strongly staining gels of samples from adjacent ages. Minor components deduced in this manner, are represented by a dashed line in fig.(62).

No difficulties were experienced in obtaining reproducible results from embryos of 4-days development and older (figs. 60 and 61). This is in contract to electrophoretic analysis of lens extracts on urea polyacrylamide gels, where embryonic ages earlier than about 7½ days could not be studied because of variation in electrophoretic mobility of components (Truman et al. 1972a).

Clearly changes occur in the relative intensities of the bands on the gels and there is a gradual increase in the number of subunits, as detected by this method, during development. The number of components detectable in the 4-day-embryo is remarkably high. For example, of the 5 major delta-subunits (see Chapter 8) only component 4 (henceforth designated as delta-1) is apparently absent, first appearing in trace amounts in 7 day-embryos. Both major alpha subunuts (previously designated as components 1 and 2) are present at this stage. In fact alpha-2 vies with delta-2 as the most intensely staining subunut in all the earliest stages of development. By day 14 most of the polypeptides which form the crystallins of the adult lens are detectable by the gel electrofocusing method. However, it may be more meaningful at this point to compare the ontogenic appearance and behaviour of the subunits in their separate crystallin classes.

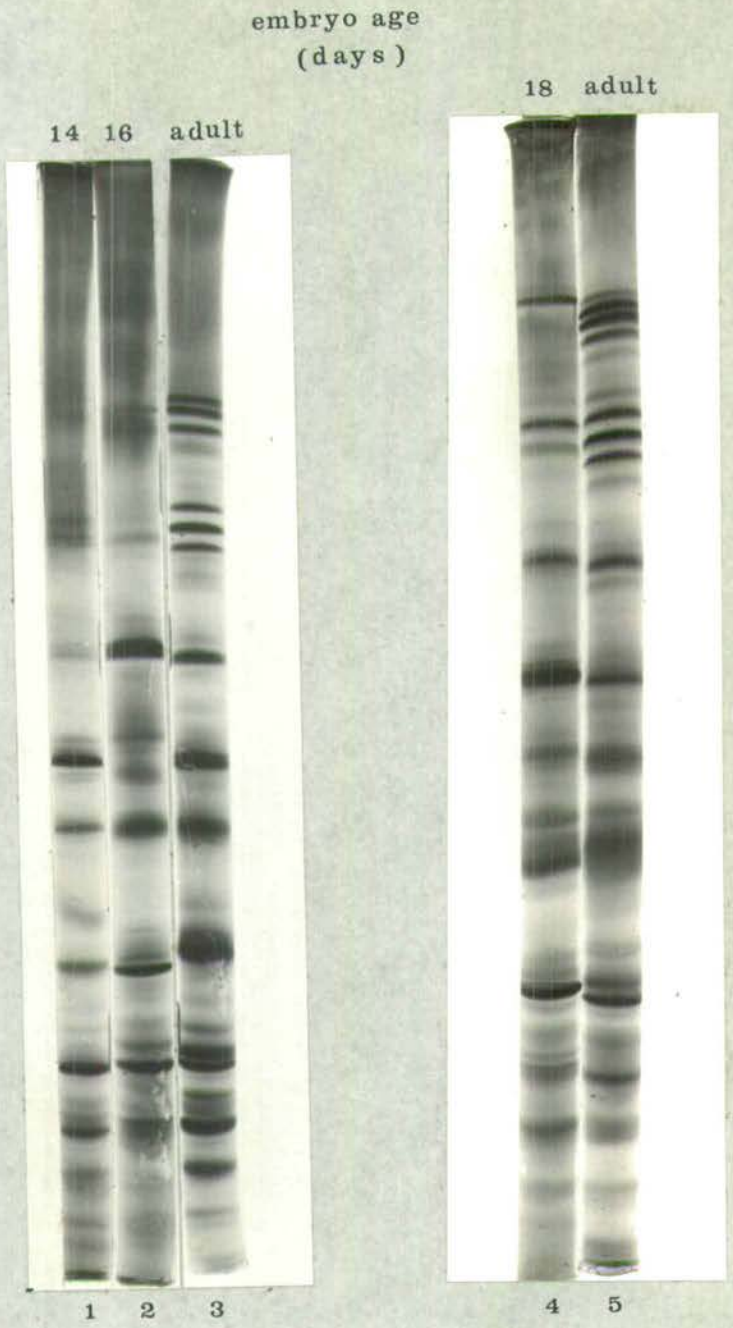


Figure 61. Isoelectric focusing of older embryonic lens extracts on polyacrylamide gels of 6M urea. Embryonic ages analysed together in the same run are shown together with an adult sample of total chick crystallin (250µg).

delta-crystallin

Of the five major components attributed in this investigation to be subunits of delta-crystallin, four are present in the 4 day-embryo. One of these delta-2 (previously designated as component 5) represents a major subunit throughout the earliest stages of development, but is rivalled in intensity in the older embryos (of 14 days and older) by delta-1. The ontogeny of this matter component is particularly interesting since it indicates a change in the proportion of delta-crystallin during development. It can be discerned first as a very faint component in the 7 day-embryo but only appears in any quantity as late as the 11 day-embryo. By 22 days of development (1 day post hatch) it has accumulated to such an extent that it represents the widest and most intensely staining component in the gel.

The subunits delta-3 and delta-4 (components 6 and 7) are also present in the 4 day-embryo and show a gradual increase in staining intensity that by 18 days of development is comparable to that found in samples from adult chicks. Delta-4 is prominent, in particular, from 14 days of development onwards.

The markedly later time of appearance of delta-1, compared to the other four major delta subunits, and their differences in relative rates of accumulation, as judged by itensity of staining, again argue that these components represent genuine subunits, rather than artefacts caused by carbamylation.

Truman et al. (1972a) found that by 3½-4 days of incubation the delta-crystallin is fully identical immunologically to delta-crystallin purified from the adult lens, so clearly any subunits

synthesised later must share immunological specificity with the older subunits. In contrast to embryonic alpha and beta crystallin and adult delta-crystallin, quantitative immunoelectrophoresis revealed that the most predominant peak, composed of delta-crystallin was asymmetrical in 5½ day-embryos (Truman et al.1972a). The authors suggested that delta-crystallin molecules of higher electrophoretic mobility may predominate in the early stages of development. Since delta-1 has a lower isoelectric point than delta-2, it is unlikely to have a lower electrophoretic mobility than delta-2, unless it is larger in size. This remains an unknown factor, but it should be noted that the rapid accumulation of delta-1 component from the 11th day of development onwards, will rapidly redress the predominance in early development of the delta-2 species. The appearance of a new major subunit will allow a number of new combinations of subunits to appear, and may well alter the overall electrophoretic properties of the undissociated complexes analysed in quantitative immunoelectrophoresis.

Alpha-crystallin

There is a marked difference in the proportions of the two major alpha-crystallin subunits. Alpha-2 (pI 5.8) is one of the predominant components of the earliest embryonic stages. It is the only band found in 3 day-embryos (gel 1, fig. 60b) although other bands may not have been detected because the sample concentration was too low. However, as judged by intensity of staining, up to the 11th day of development it is probably produced in greater amount than any other single subunit. In contrast the intensity of staining of the alpha-1 component was much less throughout the early stages of development, increasing in later

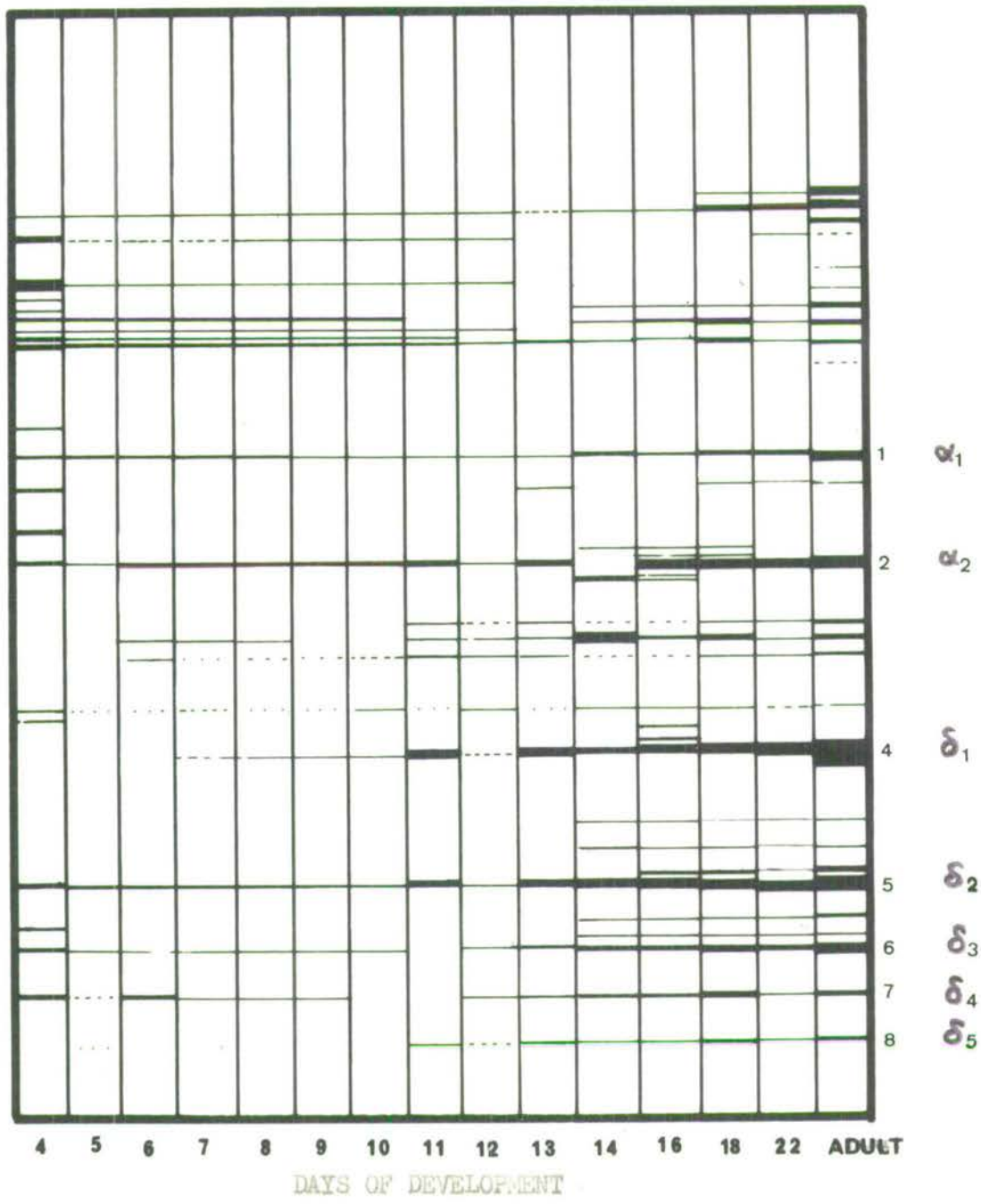


Figure 62. Diagram of the results of electrofocusing of lens extracts in the presence of 6M urea. Attributions of the bands whose radio active incorporation were subsequently followed are shown. The multiple band region of beta-crystallins (see text) proved too fine for individual isolation of components by gel slicing.

stages so that by 18 days of development the relative intensity of staining approximates to that found in adult samples (gels. 4 and 5 fig. 61). Clayton (1969) and Rana and Maisel (1969) found electrophoretic evidence of a third major subunit of chick alpha-crystallin, not present in the embryo. Using the more highly resolving system of gel electrofocusing no such third major component of alpha-crystallin was detected throughout this investigation, although it cannot be excluded that the very minor bands of similar pI to alpha-1 and alpha-2 are components of alpha-crystallin (see Chapter 6). In fact the number of different major polypeptides is unchanged after 18 days of development, and all bar one of these components are clearly detectable in 14 day-embryos. No initiation of any major polypeptide appears to take place between hatching and adulthood.

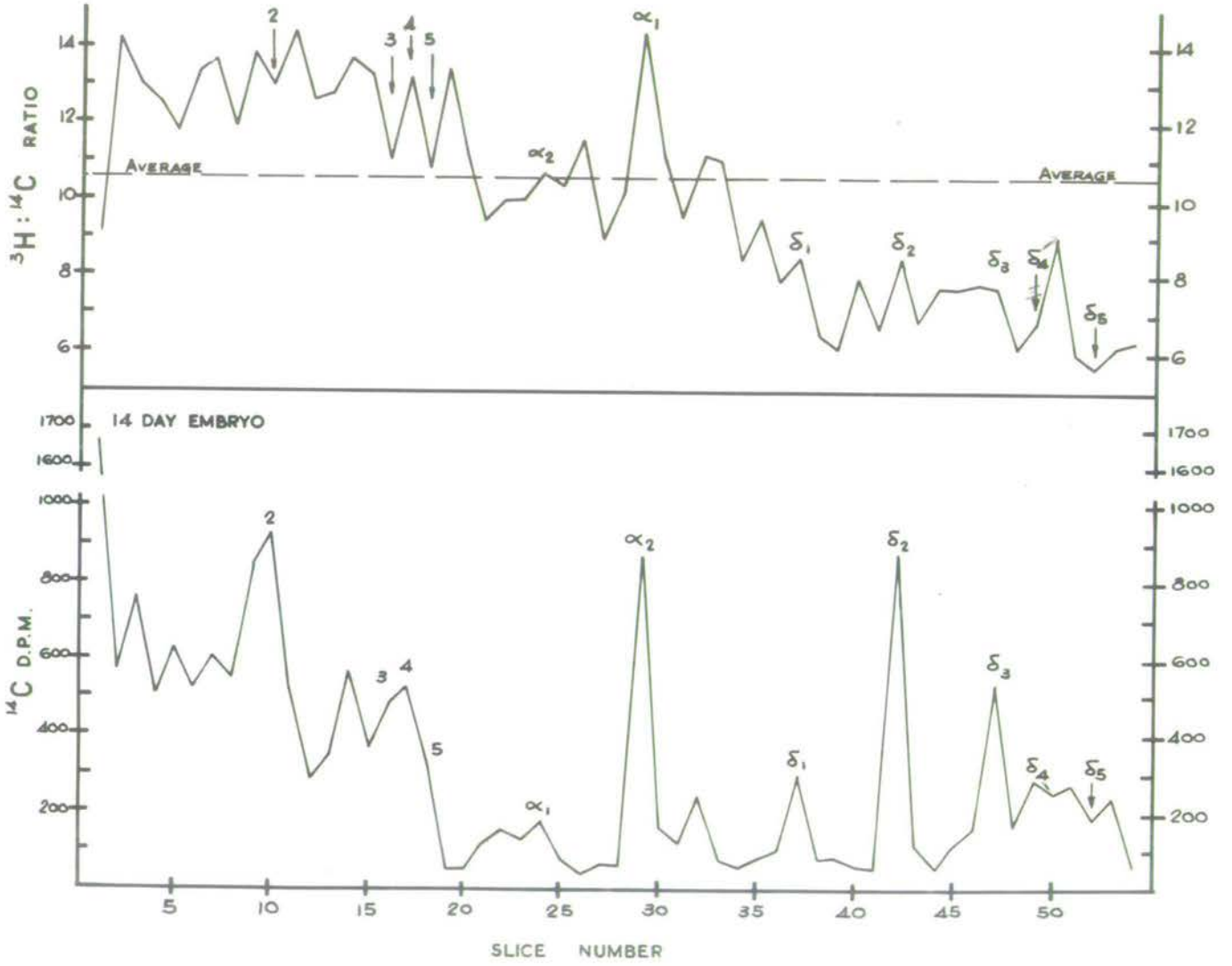
beta-crystallins

Complex changes occur in the group of beta-crystallins of low isoelectric point. A series of about eight fine bands present in the 4 day-embryo gradually come to resemble the adult pattern by about 18 days, albeit present in much smaller amounts than in the adult. These fine bands may possibly include components mainly present or restricted to outer layers of the lens, whose relative proportion will drop sharply in later embryos, where the ratio of fibre cells to epithelial cells is much higher. Extra-lenticular contamination is a less likely explanation since the complex pattern ~~obtains~~ persists until at least 13 days of development, and embryonic lenses can be easily dissected free of contaminating tissue from embryos of about 8 days onwards. (However some minor bands found only in the 4 day-embryo extracts e.g. those near the alpha-1 and alpha-2 components may derive from extralenticular

tissues). In the 4 day-embryo, beta-crystallins of low isoelectric point (probably anodal forms) appear but the beta-crystallins of higher isoelectric point (hence probably cathodal forms) are not all fully detectable by staining until about the 11th day of development. This agrees, in general, with the results of Truman et al. (1972a) who found that although cathodal beta-crystallins could be detected first, all the antigens in the anodal groups of beta-crystallins were present by 3½-4 days of development, whilst additional antigens of the cathodal group were not detectable until about 7½ days of incubation. In addition these authors found that a beta-crystallin of extremely high mobility, in alkaline conditions, (thus presumably anodal) did not appear until the 18th day of incubation. A beta-crystallin component (previously designated as D) with the lowest isoelectric point of any subunit (thus likely to have a high electrophoretic mobility in alkaline conditions) appears first in the 18 day-embryo (gel 4 fig.61). Zwaan (1963) using immmunoelectrophoresis found additional beta-crystallins appearing as late as 17 days of incubation. Since the results of the very sensitive Osserman tests described by Truman et al. (1972a) indicated that by 7½ days of development all adult crystallin antigens are apparently present in the embryonic lens then clearly components appearing later than this must share very similar immunological specificity with the members of their group already present.

It is also clear from the gel electrofocusing results that although the relative concentrations of the beta-crystallin subunits are steadily increasing, relative proportions of the antigens in these late embryonic lenses are still considerably less than those

Figure 63. Ratio counting of chick crystallins labelled in culture. Control (^{14}C) and actinomycin treated lenses (^{3}H) (Materials and Methods section) were mixed, homogenised and the proteins separated by gel electrofocusing in urea. Migration is from left to right. The lower panel shows the profile of labelled control proteins, and the upper the $^{3}H/^{14}C$ ratio. A high ratio indicated a protein whose synthesis is relatively unaffected by actinomycin. Numbers 2-5 refer to the positions of major beta-crystallins of low pI found in the adult sample, but these regions are much more complex in embryo samples.

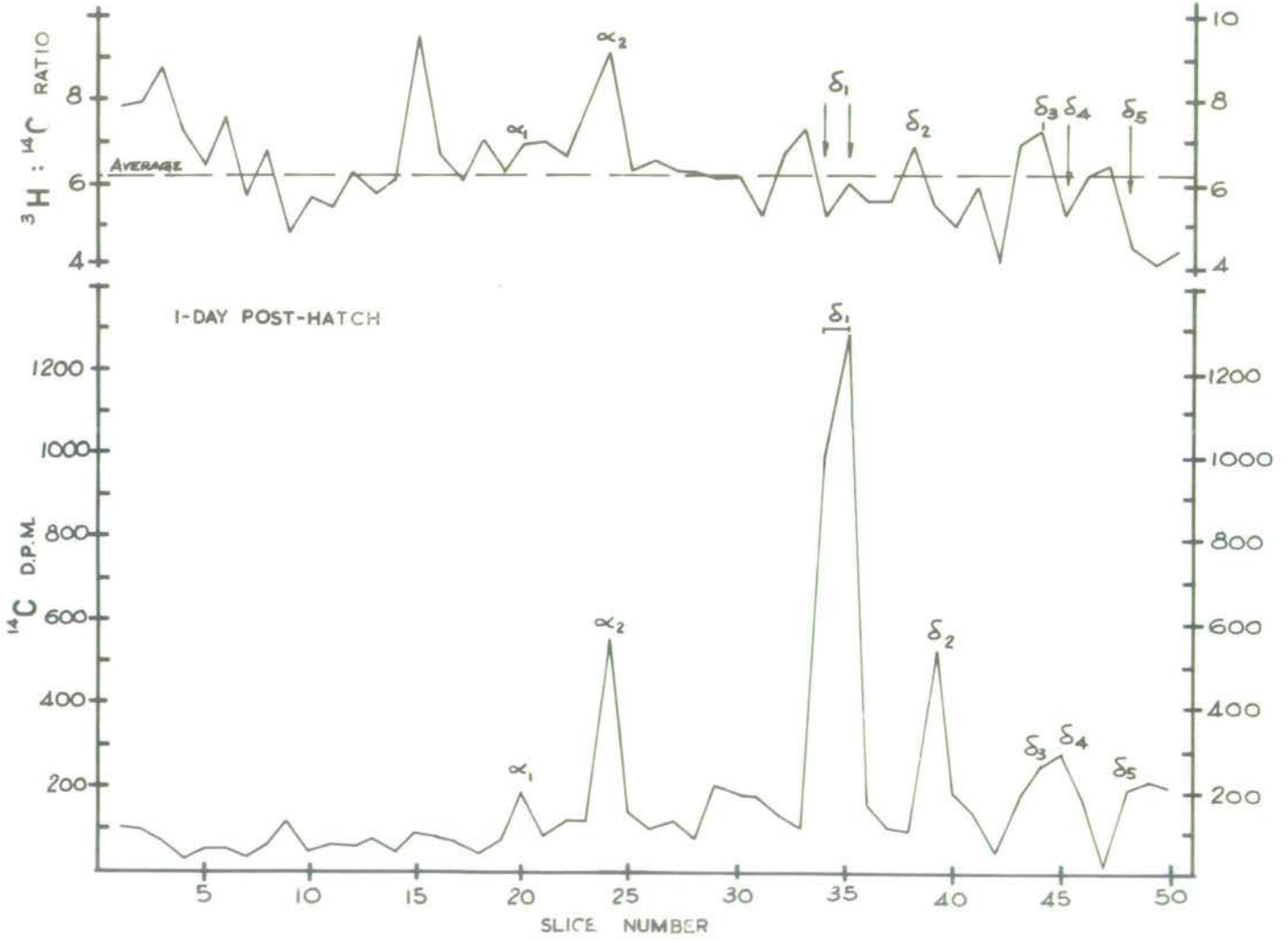


of the adult (compare gels 4 and 5, fig.61). As detailed fully in the Introduction, a steady increase in the proportions of beta-crystallin components occurs after hatching (e.g. Rabaey,1962; Genis-Galvez et al., 1968b; Truman et al.,1972a). Although showing a marked difference in intensity of staining, the similarity of the isoelectric pattern of the beta-crystallins in late embryo stages and adult samples, substantiate the conclusion of Truman et al. (1972a) that such changes are brought about by continuation of the synthesis of the same major subunits rather than the initiation of the synthesis of any additional major polypeptides.

Analysis of radioactive incorporation patterns

The experimental rationale chosen was that described by Kafatos (1972) in the investigation of a differentiated insect tissue, the silkmoth galea. Equal numbers of lenses were cultured for 4 hours, with and without actinomycin D at 30µg/ml. They were then labelled simultaneously for 4 hours, but with different isotopes: the experimental (actinomycin-treated) with 3H amino-acids at 150 µCi/ml. and the control lenses with 14C amino-acids at 15µCi/ml. In order to minimise variability introduced by separate homogenisation procedures, precise reproductibility of electrofocusing runs, etc., the two sets of lenses were washed, combined and frozen in liquid nitrogen. The ^{14}C and ^{3}H proteins were then extracted and co-electrofocused, hence the $^{3}H/^{14}C$ ratio in each gel slice gave an indication of the effectiveness of actinomycin in suppressing the synthesis of the corresponding protein. Provided actinomycin D affects protein synthesis by regulating the availability of new mRNA, then a high $^{3}H/^{14}C$ ratio would indicate a protein produced on a mRNA molecule of high stability, conversely a low $^{3}H/^{14}C$ ratio would indicate an unstable mRNA.

Figure 64. Ratio counting of 1 day post-hatch lenses, labelled in culture. The ^{14}C (control) and $^{3}H/^{14}C$ ratio profiles are shown from an actinomycin experiment similar to that described in the previous figure. Because of the complexity of the multiple beta components in the upper regions of the gel, more than one component may be present in individual slices. Hence only the ratio profiles of the major alpha and delta crystallin subunits could be reliably followed.



The counting efficiencies of both isotopes in each slice were determined automatically by reference to a series of quenching co-efficients programmed into the scintillation counter (see Materials and Methods section). For each slice both the ^{3}H and ^{14}C CPM readings were converted into DPM, and the $^{3}H/^{14}C$ DPM ratio printed out automatically. The slicing pattern of each stained gel was accurately recorded, hence different electrofocusing runs could be compared to both photographs of the stained gels and the ^{14}C DPM (control) profile.

Ratio counting of whole embryonic protein extracts

Fig. (63) shows a typical mRNA decay experiment employing actinomycin D. The lower panel represents the proteins synthesised by the control cells of 14 day embryonic lenses. The major peaks are designated according to the subunit identification scheme previously derived (see Chapter 8). The upper panel shows the isotope ratio. The synthesis of the alpha$_2$ subunit in particular appears to be relatively unaffected by actinomycin; as indicated by its high $^{3}H/^{14}C$ ratio. However much of the radioactivity entering the gel appears in the first 10 slices or so. This material did not stain for protein with Coomassie Brilliant Blue, and its origin remains obscure. The ^{14}C DPM profile of lenses from one day old post-hatch chicks reveals a much lower level of incorporation of material in this region (fig.64). The incorporation levels in this region varied from age to age, without apparent pattern, over the range of 7% to 40% of total incorporation. It may represent free amino acids incompletely removed by 10% trichloro-acetic acid during the protein staining procedure or represent membraneous material or material adsorbed onto membranes during homogenisation. Since such radioactivity could not be presumed to

Table 16. Ratios of overall incorporation in urea electrofocusing gels used to fractionate lens crystallins labelled in culture.

Age	^{3}H D.P.M.	^{14}C D.P.M.	$\frac{^{3}H}{^{14}C}$
2 day embryo	6,060	2,807	2.16
3 " "	5,689	2,581	2.20
4 " "	126,887	20,136	6.30
7 " "	45,125	7,272	6.20
8 " "	24,247	3,391	7.15
10 " "	151,434	13,021	11.63
11 " "	146,542	11,486	12.76
13 " "	111,853	12,233	9.14
14 " "	133,806	12,663	10.57
16 " "	95,674	12,853	7.44
18 " "	127,661	14,551	8.77
22 (1 day post-hatch)	50,940	8,346	6.10

Lenses exposed to actinomycin treatment were labelled with ^{3}H amino acids, whilst matched control lenses were labelled with ^{14}C amino-acids (for labelling details see Material and Methods section). All lenses were then mixed and homogenised together prior to protein extraction. Polypeptides were separated by electrofocusing in urea-polyacrylamide gels (Chapter 3). After gel sectioning the total incorporation of ^{14}C and ^{3}H isotopes were summed for all gel fractions bar the first 10 slices (see text).

be due to genuine incorporation into polypeptides, counts in this region of the gel (first 10 slices) were discarded in subsequent calculations of total DPM incorporations. It should be noted that the $^{3}H/^{14}C$ ratios of these slices were similar to, or slightly higher than, the final average $^{3}H/^{14}C$ ratio of the remainder of the gel, hence the degree of labelling of this unknown material varied little from that incorporated into the crystallin subunits, suggesting that this material may be free amino acids.

The average $^{3}H/^{14}C$ DPM ratio varies considerably with age (Table 16). The highest ratio found was that of 11 day-embryo lenses and the gradual increase in ratio up to this point could be interpreted as evidence for a gradual stabilisation of mRNA during this period, always provided that resistance to actinomycin treatment represents evidence for a stable $_{m}$RNA. However the subsequent variation and general drop in the average $^{3}H/^{14}C$ DPM ratio in later developmental stages suggests that such a simple interpretation is not tenable. The changing ratios during ontogeny of the major subunits follow the average $^{3}H/^{14}C$ of each developmental stage (fig.65). Thus the isotope ratios of the alpha-2 subunit throughout ontogeny shows a similar but higher profile to that of the average $^{3}H/^{14}C$ ratios. The isotope ratios of the alpha-1 subunit are not so markedly higher but still closely follow the overall pattern of the average $^{3}H/^{14}C$ ratios. It is difficult to accept that mRNAs apparently highly stable at 11 days of development, as judged by a high isotope ratio, subsequently become much less stable. It seems much more likely that the degree of labelling of the intracellular amino-acid pool in the presence of actinomycin varies from age to age. Slight differences in labelling

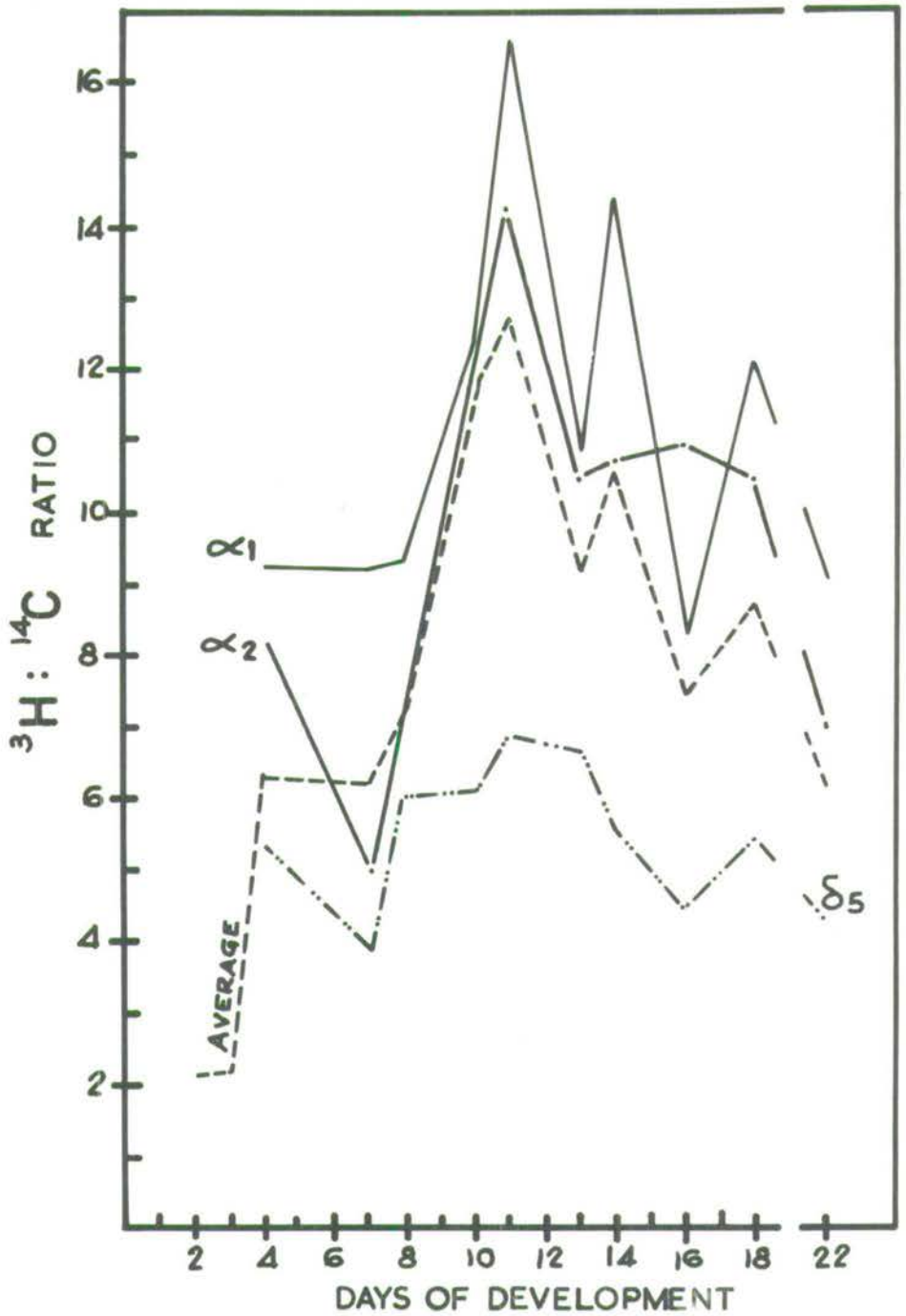


Figure 65. Comparison of isotope ratios of specified crystallin subunits, at various stages of development, with the average 3H/14C ratio, (ratio of total incorporation), during ontogeny. Note that whilst the delta-5 subunit never exceeds the average ratio during the whole period of development analysed, both alpha subunits remain above the average ratio from at least the 7 day-embryo onwards.

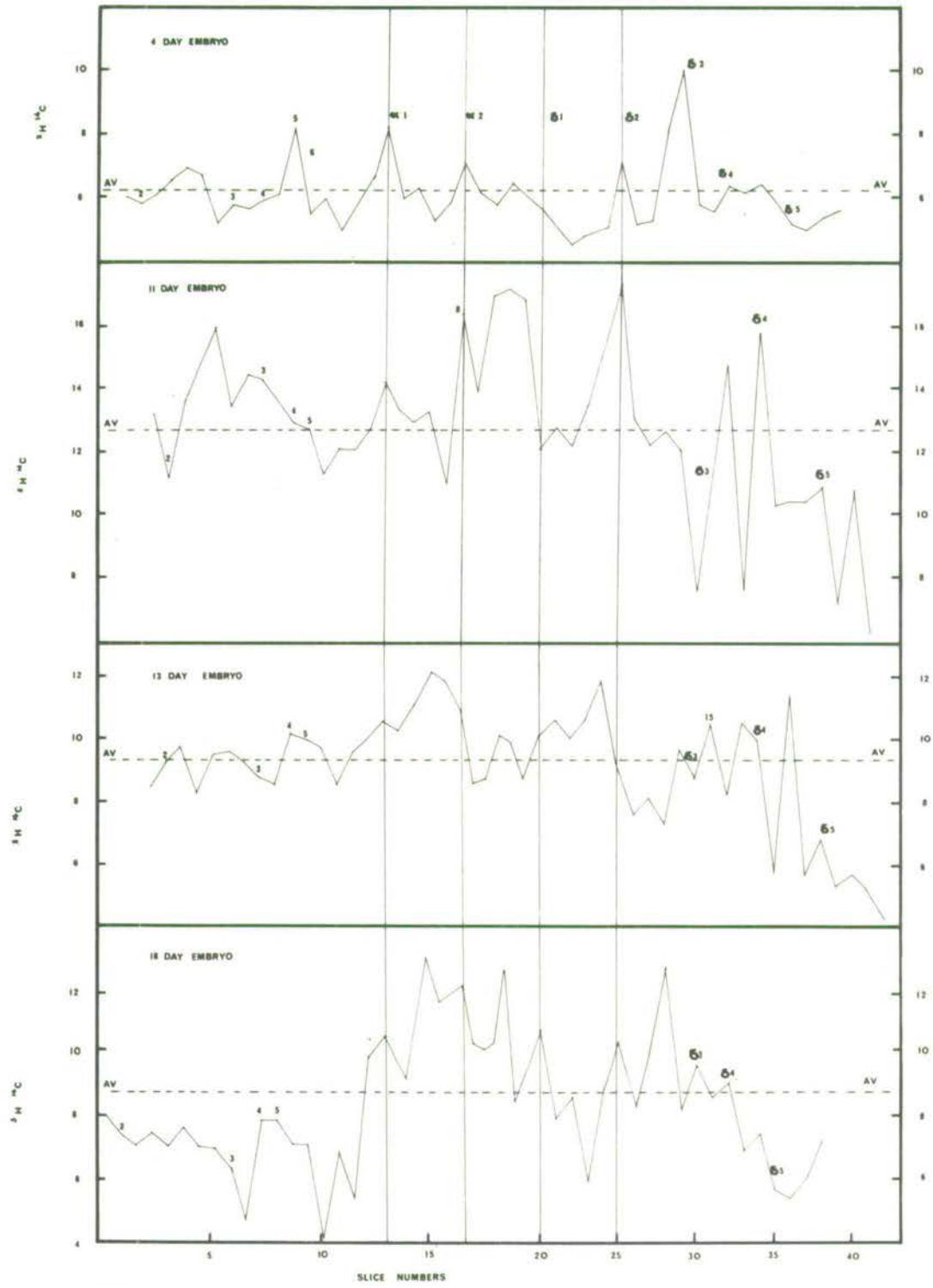


Figure 66. Variation with age of isotope ratio profiles in actinomycin. The details of preincubation in presence or absence of actinomycin, labelling conditions etc., see Materials and Methods section. Because of slight variations in gel lengths and gel sectioning, for ease of comparison, the profiles have beendrawn with the position of several of the major bands aligned. High 3H/14C ratios indicate polypeptides whose synthesis is relatively unaffected by actinomycin.

conditions, variation in the amount of control and actinomycin treated tissue, changes in proportion of epithelium and fibres etc. could lead to small differences in average $^{3}H/^{14}C$ ratios but could not account for the gross differences in ratios observed here, nor the general drop in isotope ratio in later developmental stages. Rather these factors will impose additional variation on a more general effect of changes in the intracellular amino acid pool.

The major peaks and valleys of the ratio profiles for different developmental stages did not vary widely from the average $^{3}H/^{14}C$ ratio for each particular stage (fig.66) The degree of ratio variability was much less in slices of high DPM, far greater variation from the average isotope ratio was found in regions of very low DPM where only minor differences in counting efficiency can lead to great fluctuations in final isotope ratio.

This considerable restriction in ratio values around the average, at least for the major subunits, rather than the expected pattern of persistent major peaks and valleys in the ratio profile, argues that the final $^{3}H/^{14}C$ ratio for any region is not an absolute measure of mRNA stability. The size of intracellular amino acid pools will vary for different experiments and from age to age. Kafatos (1972) points out that changes in cell position during development may affect the rate of precursor uptake into the cells; the permeability of the cell membranes may change with time, and the pool size can alter because of changes in the relative rates of protein degradation and synthesis. Moreover the intracellular amino acid pools of actinomycin treated and control cells will not be comparable. Protein synthetic and degradative rates will be different, the uptake of amino acids in actinomycin treated cells

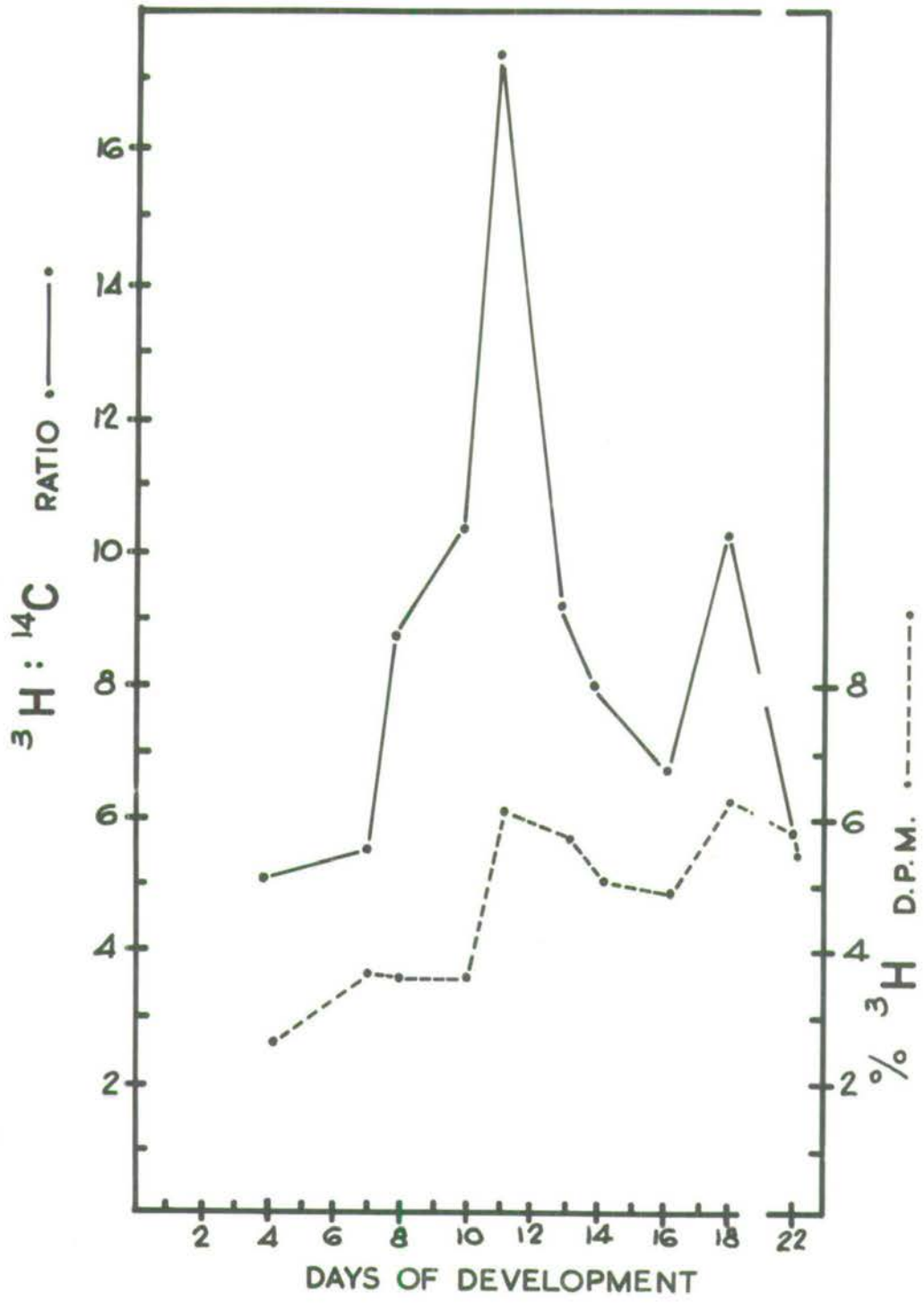


Figure 67. Comparison of isotope ratios of the chick delta-2 crystallin subunit during development with its relative rate of synthesis (expressed as a percentage of total gel incorporation) in actinomycin treated cells.

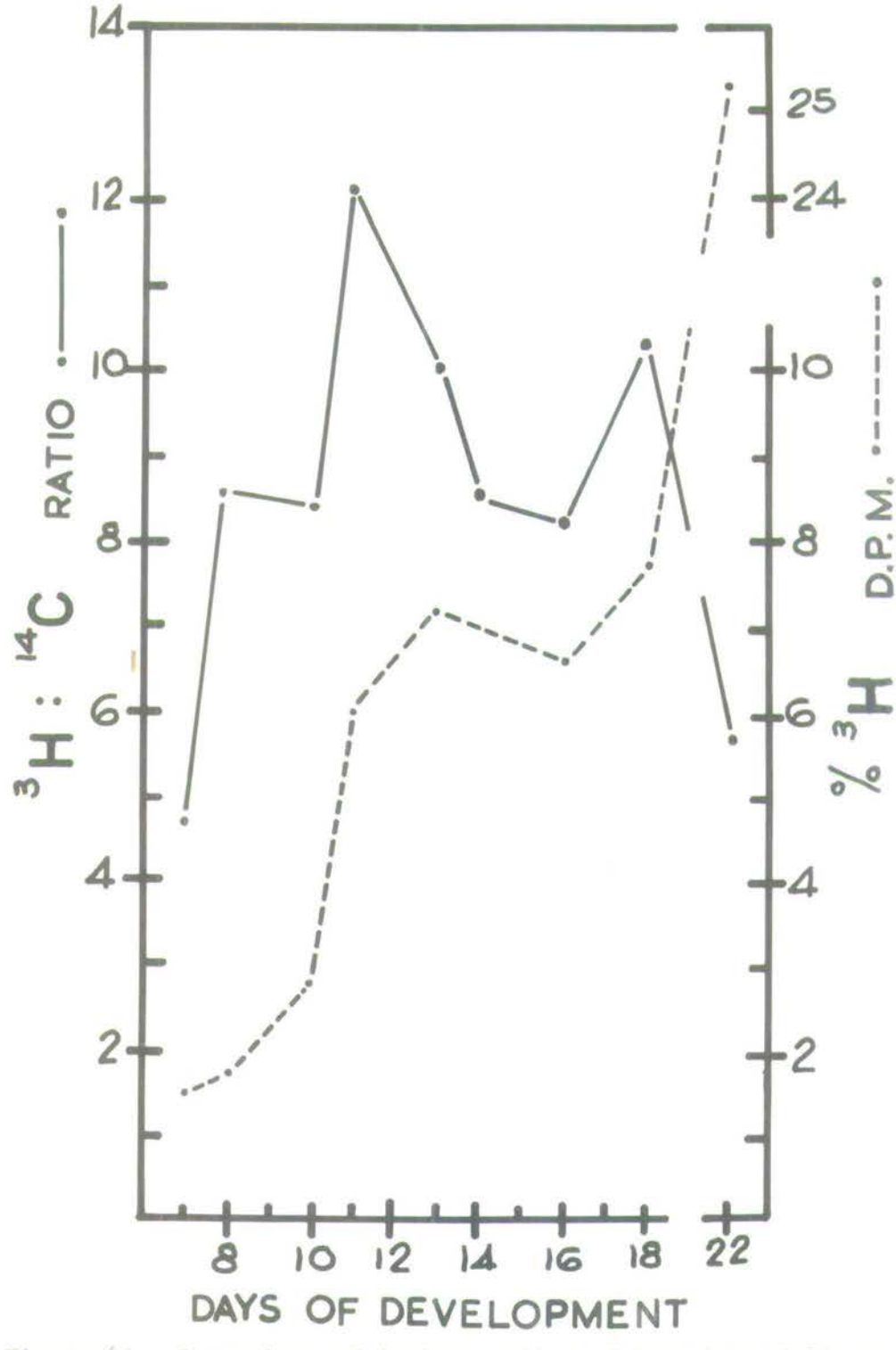


Figure 63. Comparison of isotope ratios of the chick delta-1 crystallin subunit during developent with its relative rate of synthesis (expressed as a percentage of total gel incorporation) in actinomycin treated cells. Note the rapid accumulation of this product from its first appearance in the 7-day embryo (see figure 62).

will be concentrated into those proteins whose synthesis is least affected by the action of the antibiotic. Side effects of the antibiotic, such as changes in membrane permeability, may greatly affect the final size of the intracellular permeability. For example, in the galea cells of the silk moth, actinomycin increases the specific activity of intracellular amino acids with time (Kafatos, 1972).

Direct evidence that the final $^{3}H/^{14}C$ ratio of the polypeptides cannot be used as an absolute measure of messenger stability is shown in figs. 67, 68.

Comparing the ^{3}H incorporation into individual polypeptides with the total ^{3}H incorporation in the gel gives an overall measure of resistance of polypeptide synthesis to actinomycin treatment. For delta-2 synthesis, this latter ratio varies much less sharply from age to age than the $^{3}H/^{14}C$ ratio, whilst the overall trends of both ratios remain similar (figs.67). However for delta-1 synthesis (fig.68) the relative rate of synthesis in treated cells rises sharply in the later stages of development, but the expected persistent high values in the $^{3}H/^{14}C$ ratio do not occur.

To summarise, conversion of isotope ratios into meaningful comparisons of messenger half-lives is considerably complicated by the need to compare intracellular amino acid pools of control cells, which will themselves vary from stage to stage and separate experiments, with the even more variable amino acid pools of actinomycin treated cells. Major contributions to this latter variation include changes in the relative rates of synthesis and degradation of actinomycin sensitive and resistant components and side effects of the antibiotic.

Nonetheless qualitative differences in the synthetic patterns

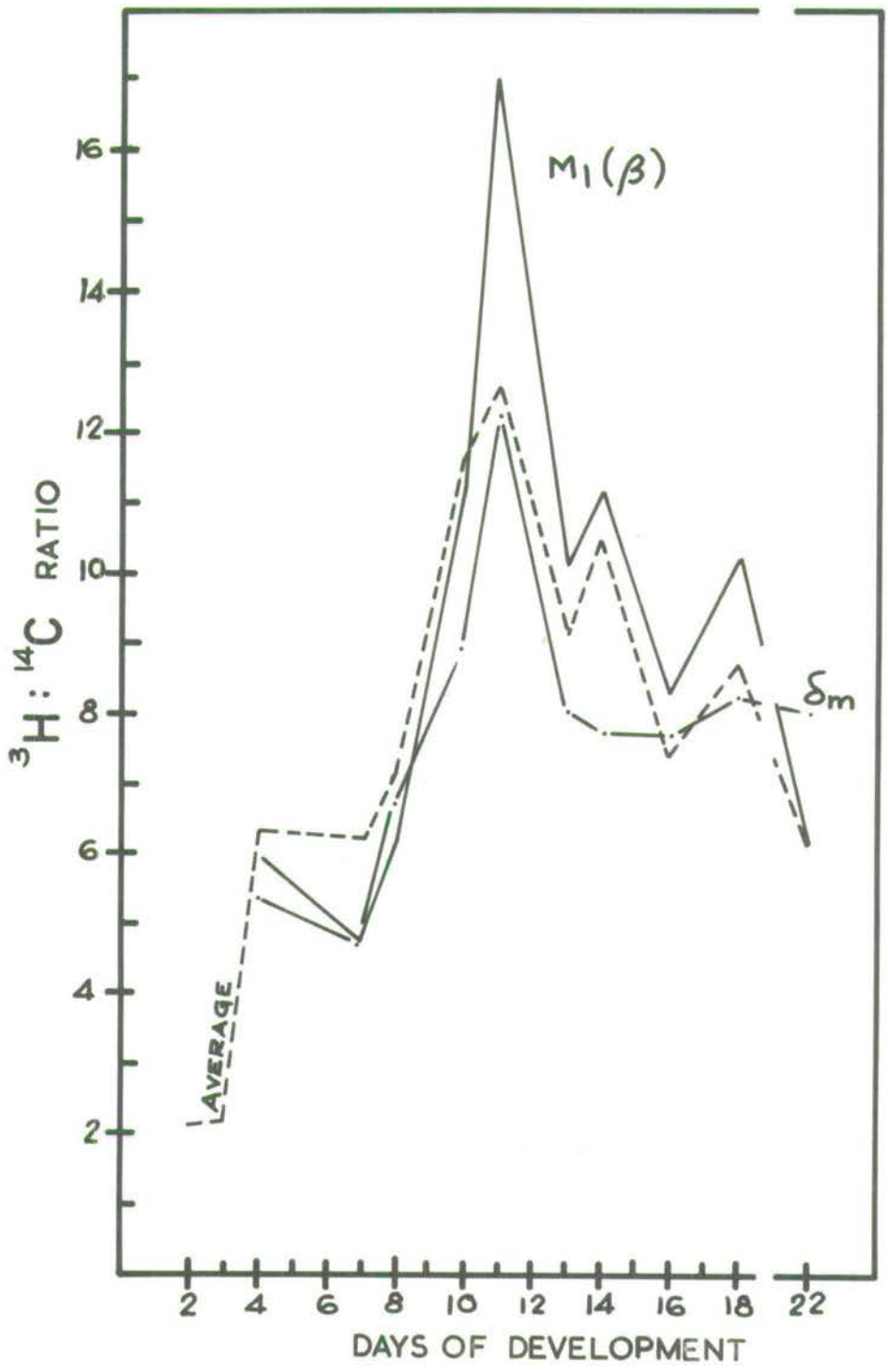


Figure 69. Comparison of isotope ratios of specified crystallin subunits at various stages of development with the average 3H/14C ratio (total incorporation into the gel). The beta-crystallin exceeds the average ratio on day 11, the delta-crystallin component only exceeds this ratio in the 1 day post-hatch chick.

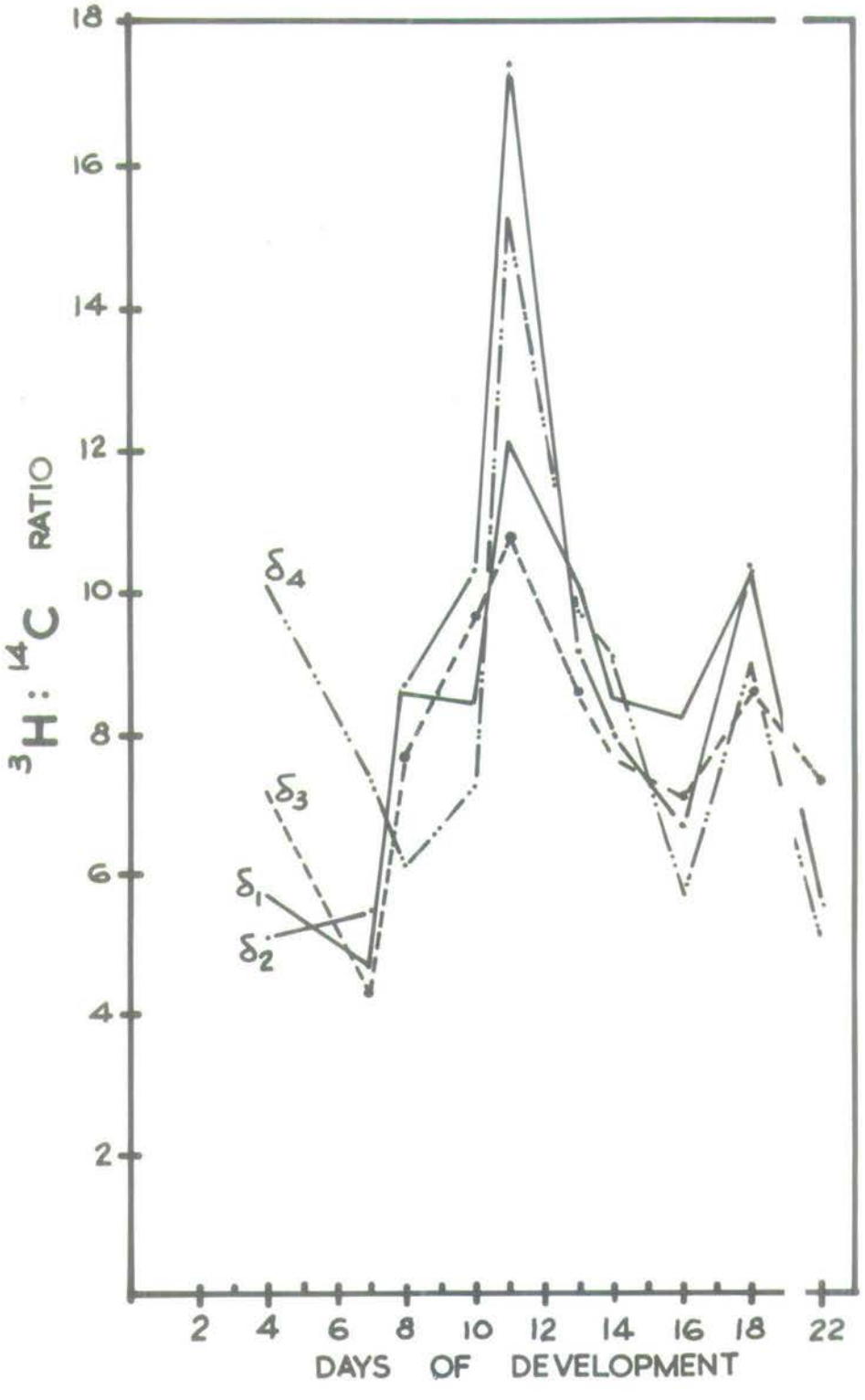


Figure 70. Comparison of isotope ratios of delta-crystallin subunits at various stages of development. Note that no one component shows a ratio consistently higher or lower than the others.

of components from actinomycin treated cells can still be discerned by comparing the isotope ratios of individual components with the average $^{3}H/^{14}C$ ratio (total incorporation in the gel). As outlined above this latter ratio is only a very general guide, being subject to considerable experimental variation, but does give a rough comparison of the degree of labelling of intracellular amino acid pools of experimental and control cells. As first shown in fig.65 the isotope ratio profiles of both major alpha subunits were consistently higher than the average ratio, which can be taken to indicate that their mRNAs are particularly stable throughout the period of development analysed. In contrast the isotope ratios of the delta-5 subunit were invariably below that of the average isotope ratio for total incorporation, suggesting that its mRNA remains unstable even after some 22 days of development (fig.65).

Other components show progressive rises in isotope ratios compared to the average ratio. Thus, the component M_1, identified as a beta-crystallin (see Chapter 8) shows an isotope ratio profile below that of the average until the 11th day of development, from which time onwards its ratio remains above (fig.69). The minor component delta-M remains consistently below the average ratio until the 22nd day of development. This may represent evidence for the increase in stability of the mRNA of these two components from the 11th and 22nd day of development respectively.

The isotope ratio profiles of the delta components 1-4 are shown in fig.70. These components in general follow the overall profile of the average isotope ratio (see previous figure) but fluctuation slightly above and below the average for each different

Table 17. Comparison of isotope ratio patterns, during ontogeny, of individual crystallin polypeptides. (The average $\frac{^3H}{^{14}C}$ ratio was calculated as described in the legend to Table 16).

Subunit	Remark (s)
Alpha-1	Invariably above average isotope ratio from at least 8 day embryo
Alpha-2	Invariably much higher than average from 4 day embryo onwards
beta crystallin M	Only consistently above average from 11 day embryo onwards
delta M	Consistently below average until 22nd day of development
delta 1-4	Constant fluctuation around average isotope ratio
delta 5	Invariably below average isotope ratio

age was found for all four components. Thus there appeared no simple pattern of ratios rising or falling below the average isotope ratio. As stressed above the average isotope ratio is not an absolute measure, being subject to experimental variation, and since these components in themselves constitute a considerable proportion of the total incorporation into the gel, then fluctuation of their ratio values around the average isotope ratio is not unlikely. The overall similarity in ratio profile of these 4 components may indicate similar mRNA stabilities.

Unfortunately any inference of mRNA stability must be indirect, being based on the synthesis of the corresponding crystallin subunit. However the results described in this section (summarised in Table 17) whilst emphasising the need for care in interpreting directly, results obtained with actinomycin, still indicate that differential mRNA stability may occur in the lens, rather than gradual stabilisation of all messengers during the course of fibrogenesis. Thus, delta-crystallin components 1-4 appear relatively stable whilst delta-5 remains unstable, yet all are prominent components of the oldest fibres. Complication of labelling introduced by actinomycin, on such factors as amino acid pools, intracellular isotope concentration, provided they affect the synthesis of all polypeptides similarly, should not invalidate relative estimates of mRNA stability. This is not the case if the rate of synthesis of particular polypeptides are controlled by specific factors subsequently affected by the antibiotic (Kafatos, 1973). This point is examined again in the Discussion section.

Chapter 15

DISCUSSION

Often the study of synthesis of specific cell proteins by terminally differentiated cell types offers the advantage that these proteins are made in large amounts. Because of their abundance the proteins are usually easy to purify. Unfortunately whilst some 80 to 90% of the total protein content of the chick lens is composed of the structural proteins, the crystallins, purification of protein fractions in good yields of material has proved considerably more difficult for the components of this species than mammalian lens proteins. Neither polyacrylamide gel electrophoresis (Chapters 4 and 5) nor gel filtration techniques (Truman, 1968) have, to date, produced entirely satisfactory levels of resolution, particularly of undissociated components. However the development of a gel electrofocusing technique offers, because of its high degree of resolution and reproducibility, a suitable analytical technique for investigating the ultimate gene products, the crystallin subunits.

It is significant that other workers, entirely independently, developed a very similar gel electrofocusing technique, employing dissociating conditions, for analysing mammalian crystallins (van Kamp et al. 1974 a,b). Hoenders group employ dithiothreitol (DTT) to avoid oxidation and dimerisation of subunits (rather than 2 mercaptoethanol) and ampholytes of pH 5-8 (Liem-The and Hoenders, 1974b) but the overall method is highly similar to the technique described in Chapter 3, both methods being developed from the method of Wrigley (1968). This group found that the resolution of subunits by electrofocusing is markedly improved compared with gel electrophoresis in 6M-urea (Liem-The and Hoenders, 1974a) in agreement with the results described in Chapter 4. Thus in electrophoresis the authors were not always able to discriminate between the various subunits of the crystallins. In particular the basic subunits of bovine alpha-crystallin

(B_1 and B_2) showed the same electrophoretic mobilities as certain components of both beta and gamma crystallins. The polypeptide B_x of bovine alpha-crystallin, revealed by gel electrofocusing, is found only in prenatal lenses, (van Kamp et al. 1974A). It probably represents a degraded chain of the primarily synthesised product, which in older lenses appears to be also converted, by deamidation, into a second product, the B_1 chain (van Kleef et al. 1974). Van Kamp et al. (1974a) point out that these two polypeptides, B_x and B_1, cannot be resolved by the basic polyacrylamide gel electrophoresis employed by Palmer and Papaconstantinou (1969) and Delcour and Papaconstantinou (1972). These independant results on the prenatal bovine lenses emphasise again the conclusion reached in Chapter 4, that the appearance of a single band in urea electrophoresis does not necessarily indicate evidence for isolation of separate subunits. Since electrophoretic mobilities of crystallin components determined by both charge and molecular weight, so often overlap, the need for a separation technique based upon a single parameter, such as iso-electric point, becomes obvious.

Most of the work characterising the isoelectric points of crystallin subunits has been performed on species other than the chick. Truman and Clayton (1974) analysed cortical preparations of chick lenses rich in beta-crystallins by column iso-electric focusing in the presence of 7M-urea over the pH range 5-8, at 17°C. Comparison of results is difficult since measurements at this temperature will be some 0-I - 0.3 of a pH unit lower than at 4°C (see Chapter 3). In addition the authors reported that the temperature regulation of the column was not precise. Even so, several components of high pI appear to be outside the beta-crystallin pI range found in this investigation. The reason for this discrepancy is not known. Liem-The and Hoenders (1974a) also found a more restricted range of pI values of rabbit beta-crystallin subunits. Of three beta-crystallin fractions obtained by gel filtration, containing a total of about 13 subunits,

two fractions included components between pH 5.9 and 7.0, the third between pH 5.4 and 7.0. 7-8 major bands between pH 7.0 and 7.8 were found for gamma crystallin.

Alpha-crystallin subunits from rabbit and bovine lenses showed very similar iso-electric points (Liem-The and Hoenders, 1974a). In rabbit lenses the two acidic proteins (alpha A_1 and alpha A_2) have isoelectric points of 5.7 and 5.9, and the two basic proteins (alpha B_1 and alpha B_2) of 6.5 and 6.9 respectively (taken from data of Liem-The and Hoenders 1974a). Corresponding values for subunits of bovine alpha crystallin are 5.6, 5.9, 6.6 and 6.85. These values for the pI of the two basic proteins differ from earlier estimates of approx. 7.1 and 7.4 (Palmer and Papaconstantinou, 1968; Schoenmakers and Bloemendal, 1968). It has been suggested that the acidic subunit alpha A_1 is formed by post-transcriptional modification of the primary structure of subunit alpha A_2 (Palmer and Papaconstantinou, 1968, 1969; Delcour and Papaconstantinou, 1970). A similar claim has also been made for the origin of the alpha B_1 subunit from alpha B_2 (Stauffer et al., 1974).

Other minor alpha crystallin components found in the nucleus are now known to be degradation products already formed in the early stages of development. (Liem-The and Hoenders, 1974b).

Hence the A_2 and B_2 chains appear to be the fundamental polypeptide chains. Thus comparison of the number of alpha-crystallin chains in bovine and chick lens is complicated by the intracellular degradation and conversion processes of the bovine alpha-crystallin. However since only two major alpha-crystallin subunits (pI 5.4 and 5.8) were found in the chick (see Chapters 5 and 8) the number of fundamental polypeptide chains may be similar in both species. Since the pI of a polypeptide represents the ratio of positively charged side-groups to negatively charged side-groups, comparatively few substitutions of one type of charged amino-acid by another

of opposite or neutral charge, during evolution, are need to considerably alter the final pI of the polypeptide. Modifications in amino-acid sequence in mammalian lenses appear to have been stringently conserved. This is shown in their very similar immunological reactions (Bjork, 1968) and similar pI (Liem-The and Hoenders, 1974a). Clayton (1974) pointed out that many of the reported substitutions in αA_2 chains of mammalian species would not affect conformation.

The variety in subunit structure of bovine alpha-crystallin was not found in the work reported here on chick alpha-crystallin. This species difference is of interest since the phenomena of specific and limited degradation of bovine alpha crystallin appears absent in the chick stain analysed here. As described above it now appears that many of the calf lens ^may represent degradation products of polypeptides produced in the embryo, and studies on protease activity reflect this view. Van Kamp et al., (1974b) could detect no leucine amino-peptidase activity in bovine embryonic lenses younger than 3 months. The initial rate of synthesis at 3 months was high but decreased after about 6½ months of development. In the 14 week old calf, the activity of this peptidase was confined to the nucleus and cortical regions of the lens (van Kamp and Hoenders, 1973). Since its activity was greatest in the outer layers of the cortex the authors suggest that the enzyme may also be involved in the destruction of the protein synthesising apparatus, a characteristic of fibrogenesis (see Introduction). Differences in the complexity of alpha-crystallin between chick and bovine samples may merely reflect the great differences in development rate of the two species. Degradation appears to occur on too slow a time scale to be detected in the chick strain employed here, which reaches adulthood within a few weeks. It seems more likely that the degradation represents an uncontrolled aging process rather than a regulatory mechanism used to keep

the lens proteins soluble and the lens transparent (van Kleef et al. 1974).

One aim of determining the subunit structure of the chick crystallins was to derive an estimate of the number of separate loci active during the development of the lens. In chapter 9 the analysis of chick crystallin subunits by both gel electrofocusing and SDS-poluacrylamide electrophoresis were compared, and the gross number of components shown to be comparable. One major point arising from these studies was that earlier work on the molecular weight of chick beta and delta crystallins may have given underestimated values (Truman et al., 1971). The major component was found to have a molecular weight of 46,000 in agreement with Piatigorsky et al. (1974) and this component has since been demonstrated immunologically to be delta-crystallin (Milstone and Piatigorsky 1975). Hoender's group have also examined the molecular weights of rabbit and calf crystallin subunits by SDS-polyacrylamide electrophoresis (Liem-The and Hoenders, 1974a). The beta crystallin of rabbit lenses revealed four bands with molecular weights between 23,000 and 32,000, beta-2 crystallin five bands between 23,000 and 39,000. Beta-3 crystallin showed three major bands with values between 22,000 and 36,500, and two minor components (barely visible in photograph) of 17,700 and 16,000. The values for the beta-crystallins of the calf ranged between 23,000 and 28,500. These authors also estimated values of 21,000 for rabbit alpha-crystallin, 20,000 and 22,000 for calf alpha-crystallin and values of about 20,500 and 21,000 for the gamma crystallins of rabbit and calf respectively.

These estimations are in excellent agreement with the general range of molecular weights of the chick polypeptides described in Chapter 9. Thus the most recent evidence, taken together with that cited in Chapter 9, indicates that the range of molecular weights of crystallin polypeptides in such diverse species as chicks and rabbits are comparatively similar.

This can be readily understood if stringent selection pressures operate on the lens peptide chains (Day, 1971; Clayton, 1974). This finding is in contrast to earlier estimates of molecular weight obtained by gel filtration which suggested provisional values of 25,600 and 16,500 for chick delta and beta subunits respectively. The results of Liem-The and Hoenders (1974a) and Piatigorsky et al., 1974 substantiate the conclusion that the latter values achieved by gel filtration represent underestimates of true molecular weight.

How many separate gene products?

Estimation of the number of genes active in the lens tissue requires unambiguous identification of separate polypeptides. As detailed in the Introduction and Chapter 9 there is some controversy as to the number of different polypeptides chains which make up the delta-crystallin polymer. In SDS-polyacrylamide gel electrophoresis delta-crystallin migrates as a single component of molecular weight about 46,000 (Piatigorsky et al., 1974, Milstone and Piatigorsky 1975, Chapter 9 of this investigation). The possibility has been invoked that the separate (putative) subunit fractions derived from urea-dissociation studies may arise from side-effects of the urea rather than represent genuinly different polypeptides (Piatigorsky et al., 1974). The evidence presented here suggests strongly, however, that these separate components are not artefacts of urea dissociation. Analysis of an apparently pure sample of delta-crystallin, as revealed by immunoelectrophoresis, showed three major bands in urea gel electrofocusing, components previously detected as prominent components of fractions enriched in delta-crystallin (Chapter 5). A similar analysis of the crystallin fractions obtained by extended agarose electrophoresis revealed, in addition, two lesser components to be constituents of delta crystallin (Chapter 6). This designation was also in

accord with provisional attributions made from analysis of fractions enriched in delta-crystallin (Chapter 5). Do the separate components represent carbamylation products? Gel electrofocusing in urea is such a sensitive technique that it has been used specifically to follow the successive carbamylations of amino-acid groups in chymotrypsinogen A, denatured by urea at high temperature (Bobb and Hofstee, 1971). The consistency in isoelectric point of the crystallins argues against their being artefacts of carbamylation for this is a process dependent upon the time of exposure to cyanate ions in urea solutions, and ultimately the degree of carbamylation will be reflected in a continuous lowering of iso-electric point. Considering that delta-crystallin fractions were obtained from different separation procedures, from different batches of animals and subjected to varying times of exposure to urea, one would expect to see, if carbamylation were occuring in these experimental conditions, some occassional variance in iso-electric patterns representing intermediate stages in the process of carbamylation. Hence their consistency in pI and the failure to detect successive stages and degrees of carbamylation argue that these components are genuine, different, subunits.

Truman et al. (1971 and 1972a) chose to carry out electrophoresis of crystallin samples in 6M urea at pH 5.6, in an attempt to minimise carbamylation of the proteins, which might be entailed in the use of alkaline urea (Stark et al., 1960). Some 5 components were provisionally altributed to the delta crystallin class, all detectable in the 4 day embryo. The final pH conditions obtaining in the gel electrofocusing system employed here (range approx. pH 3.8-8.2) ensures that almost the entire length of the ampholine gel is at an acid or near neutral pH, thus conditions of alkaline urea will be minimal.

More direct evidence that delta crystallin is composed of different polypeptide chains derives from the ontogenic studies described in Chapter 14. The separate appearance in ontogeny of delta-1, the separate accumulation patterns of the 5 components, as judged by intensity of staining, and finally the differences in their rates of synthesis, cannot be explained by carbamylation of a single component. The final number of 5 major subunits revealed by the electrofocusing method is in good agreement with the less highly resolving techniques of starch gel electrophoresis (Truman et al., 1972a) and polyacrylamide gel electrophoresis (Clayton 1969).

Delta-crystallins of the chick are presumably antigenically similar since they form a single arc in immunoelectrophoresis, suggesting similar amino acid composition and indeed similar molecular conformations. The comparatively similar pIs of the 5 putative delta components also argues that their overall charges are likely to be similar. In SDS gel electrophoresis where charges are obliterated, the differences in molecular weight of the components may be too slight to be resolved as bands of separate mobility. Thus whilst remarkable degrees of heterogeneity have been shown for both rabbit and bovine alpha-crystallin, in SDS-polyacrylamide gel electrophoresis only 1 band and 2 bands, respectively, were obtained for the alpha-crystallin samples (Liem-The and Hoenders, 1974a).

These components are unlikely to be products of slow post-transcriptional modifications since all five components showed significant levels of incorporation, throughout ontogeny, after only a 4 hour pulse (Chapter 14). Such modifications of fundamental polypeptides in the bovine lens are not in any case, detected in early embryonic life (van Kamp et al., 1974b). Conceivably their radioactive incorporation could be explained if the components represent rapidly formed degradation products, with minor

sequences of amino acids removed but this would not explain their separate synthetic rates nor separate appearances in ontogeny. Moreover, this is unlikely from results found for the bovine lens, where as detailed above, protease levels of activity are extremely low or absent in the first few months of development. Ultimately sequence data will be required to confirm that these components represent genuinely separate genetic products but the bulk of evidence to date strengthens the view that delta-crystallin, like the other crystallin classes must be a family of heteropolymers, each molecule containing a number of different subunits. This concept was first suggested by Clayton and Truman (1967) and these authors have subsequently provided elegant immunological proof that the chick beta crystallins clearly consist of a number of different molecular species (Truman and Clayton, 1974, Clayton and Truman, 1974).

The final attributions of subunits to crystallin class (figure 31, Chapter 8) include 2 major alpha-crystallins, 5 major delta-crystallin components and 11 beta-crystallins, in the adult. There is some evidence (Chapter 6) that in addition some 2-3 minor bands of isoelectric point may be beta-crystallins. Comparison with the findings of other authors (fully detailed in the Introduction) reveals a surprisingly consistent picture for the final number of different polypeptides in each class, considering the variety of techniques used. Thus in this laboratory Clayton (1969) found 3 major alpha components and 5 major delta components and 11 beta-crystallin components by polyacrylamide gel electrophoresis of adult chick samples in dissociative conditions. Truman et al., (1972a) found 5 delta components in starch gel electrophoresis in urea, whilst Truman and Clayton (1974) detected about 11 peaks by column electrofocusing total beta-crystallins in the presence of urea. The final estimate of separate gene products made in this study by

the comparatively new technique of urea gel electrofocusing are thus in very good agreement with similar estimates made with less highly resolving technique. The major advantage of the electrofocusing technique lie in its reproducibility, and since it separates on the basis of a single parameter, charge ratio, the avoiding of ambiguities present in electrophoresis. With components of similar charge and molecular weight, electrophoretic mobilities may overlap, without implying any relationship between the components. A second-dimensional electrophoresis often reveals that single bands are not always homogeneous (e.g. Maisel and Goodman, 1964, Zwaan, 1968). Such overlapping of components is much less likely in electrofocusing where differences in isoelectric point of 0.02 to 0.05 of a pH unit can be resolved (Bours, 1971).

To summarise at this point, characterisation of the polypeptides of the chick lens was attempted by gel electrofocusing techniques and SDS-polyacrylamide gel electrophoresis. Quantitative data derived from this investigation is in good agreement with the work of other authors. This is true of the most recent estimates of molecular weight of crystallin polypeptides in chick and mammalian species, and of the final estimate of the number of separate gene loci active in the lens system, derived from urea gel electrofocusing. Other workers employing a similar system to investigate mammalian crystallins have also stressed that this technique is preferable to fractionation of crystallin subunits by electrophoresis on polyacrylamide gels in the presence of 6M-urea (Liem-The and Hoenders 1974a). With the development of each new analytical technique of improved resolution there is a need, each time, to re-appraise data gained from earlier analytical methods. Such work as ennumeration and comparison of subunits, although of fundamental importance, can take on the nature of a complex but uninspiring jig saw puzzle. It is hoped however that the method of analysis of crystallin subunits and the results obtained will contribute eventually to

additional aspects of lens development. Thus by using antibodies prepared to isolated subunits, fractionated by electrofocusing, it should be possible to continue the work began in this laboratory (Clayton, 1970) on the localisation of the various crystallins within the lens. Secondly it should now be possible to derive exact, quantitative data on the rates of specific subunit synthesis during differentiation, although it is clear that complexities remain in using isotopic methods for measuring these rates (Chapter 14, and Kafatos, 1972). The field of differentiation remains surprisingly poor in such quantitative data. (Kafatos, 1972, Milstone and Piatigorsky, 1975).

With regard to the analysis of the protein components of the mRNP particle released by EDTA dissociation of chick lens polyribosomes, the major conclusion reached, namely that discretepolysomal mRNA species of the lens are associated with the same mRNA-binding proteins, is supported by other authors findings, reported during the course of this work. Bryan and H ayashi (1973) using similar zonal centrifugation techniques to those described here, could separate mRNP from the dissociated ribosomal subunits of chick emryo cerebella into discrete classes. These classes were estimated by sedimentation in an isokinetic sucrose gradient to be a) 10-30s, b) 50s and c) 70-200s. Using a similar SDS discontinuous gel electrophoretic system to that used in this investigation (but employing 10% acryl amide) they found that two proteins of molecular weight 48,400 and 78,500 were common to all mRNA fractions. In addition Blobel (1973) showed that highly heterogeneous cell types such as rat hepatocytes and L cells have attached to their polysomal mRNA, two proteins of molecular weight (78,000 and 52,000) identical to those attached to rabbit globin mRNA, although the distribution of other minor proteins appeared more complex.

In essence then it seems likely that the mRNA-associated proteins are not specific to a given mRNA, but rather that discrete polysomal mRNAs within the same cell associate with an identical set of proteins. This conclusion might perhaps have been predicted, since if a given messenger has to be complexed with a highly specific set of proteins, one would require coordination of the syntheses both of the new major mRNA species and that of the mRNAs coding for the specific mRNP-proteins. If these latter messages in turn required specific mRNP proteins coded for by messengers themselves requiring a specific newly made set of proteins and so on, an infinite series of mRNAs

and specific mRNP proteins would need to be produced. Thus it never appeared possible on logistic grounds for each type of mRNA within the cell to be complexed with its own individual set of proteins, since this leads one to ask the question 'Quis custodiet ipsos custodes?' However it still appears possible that the particular protein complex associated with any given mRNA might reflect or determine the physiological state of that mRNA. Is polysomal mRNA associated with the same set of proteins as 'free' cytoplasmic or non-polysomal mRNA? Are these proteins involved in the sequestering of mRNA? Can particular combinations of these proteins or quantitative variations in the amounts of the proteins affect the rate of translation of specific mRNAs? For these particular types of problems it is possible to imagine a role for discrete, separate sets of proteins, whereby a particular population of mRNA-associated proteins could be exchanged when the mRNA is sequestered or enters the translation machinery. This type of hypothesis can be tested directly by comparing the protein moiety of the non-polysomal 'free' mRNA-protein complexes with that in the mRNP complexes liberated from ribosomes by EDTA orpuromycin. Unfortunately such evidence as there is, is conflicting.

The first analysis, carried out on duck globin mRNPs indicated that the free cytoplasmic particles were associated with a set of proteins other than those associated with polysomal mRNA (Gander et al. 1973). But this finding is in direct contrast with recent reported results although these are only preliminary results. Thus the post-ribosomal supernatant of rabbit reticulocytes contains RNA which is substantially enriched in alpha-globin messenger activity. mRNP isolated from this fraction contains the same two major proteins as the polysomal mRNA and is not removed by centrifugation through

0.5mKCL sucrose gradients (Gross, K. W. and Baglioni, C., unpublished observations cited in Gross et al. 1973). A preliminary report has also appeared that claims that the post-ribosomal particles and the polysomal mRNP of Ehrlich ascites tumour cells are both associated with two major proteins of molecular weight 78,000 and 52,000, plus 11 other minor components (Barrieux et al. 1974). The comparison of the autoradiographic profiles of the two sets of proteins suggested, in contrast to Gander et al. (1973), that the same proteins were associated with the mRNA before translation as during translation.

In consequence, although these are very preliminary reports, it means that the intriguing hypothesis that an exchange of proteins must take place before the message can be incorporated in the translation complex (Gander et al. 1973) remains unconfirmed. Rather the recent evidence suggests that when mRNA passes into the cytoplasm it becomes saturated with a set of proteins that are capable of binding to any species of mRNA, and that these proteins remain associated with the mRNA as it enters into translation. Rather than envisaging each species of mRNA being associated with a specific, individual spectrum of proteins, both as free mRNP particles and as translated, polysomal message, it seems more probable that a discrete set of proteins are attached to all the polysomal mRNA species of the cell, and that possibly these proteins are identical to those found attached to cytoplasmic non-polysomal mRNA.

These findings substantiate the results for lens polysomal mRNA reported here but in turn raise the question of how the proteins are bound to the mRNA. Do the proteins recognise specific nucleotide sequences common to all the polysomal mRNA species of a particular cell type? Alternatively the proteins may recognise some less specific

feature of the mRNAs such as the phosphate back-bone, the overall negative charge of the molecule or regions of high secondary structure. In all cases the binding appears to be of a different nature to that of non-specific adsorption of proteins onto mRNA, since it is resistant to dissociation by high ionic conditions.

DO mRNA-ASSOCIATED PROTEINS RECOGNISE SOME FEATURE OF THE mRNA LESS SPECIFIC THAN NUCLEOTIDE SEQUENCE?

Allowing for the variation in the experimental source of polysomal mRNPs, their method of extraction, and the techniques used to analyse the protein moiety of such mRNPs, it is remarkable that most authors estimate that the molecular weight of the major protein components fall into two size classes 60-80,000 and 45-52,000. (However Lebleu et al (1971) reported that the molecular weight of the components from rabbit globin mRNP are 130,000 and 68,000.) These results suggest that there may be a restriction on the size class of the proteins that can enter into tight binding with mRNA. Possibly regions of elaborate secondary structure within the mRNA would restrict effective binding properties to a particular size class of protein. New techniques in electron microscopy however, now make it possible to visualise messenger ribonucleoprotein particles (Dubochet et al. 1973) and it now seems likely that the relationship between the secondary structure of the mRNA and the positions of the protein within the RNA-protein complex will be elucidated. In both mRNP and mRNA, regions of high density (200^{o}A in diameter) alternate with much finer strands (40^{o}A in diameter), and the regions of high density disappear with the denaturation of the RNA, suggesting they are regions of high secondary structure. The proteins appear to be concentrated in four to seven points along the

RNA molecule. It remains to be answered whether the messenger specific proteins associate with specific regions of the RNA, such as those of high secondary structure, or whether the protein is evenly coated along the RNA thread, in which case the high density regions might result from the secondary structure of the ribonucleoprotein itself. There is some further evidence to suggest that the proteins associate with RNA regions that already show a high degree of secondary structure. Studies of ethidium bromide binding showed that when purified duck globin mRNA was compared with mRNP, fewer sites of intercalation were available in the mRNP, suggesting that the proteins were partially shielding the RNA from the dye. Extensive base paired regions equivalent to those found in the mRNA could still be detected in the mRNP (Favre et al. 1973). Confirmation of the finding that the proteins are concentrated in four to seven points along the RNA molecule would favour the concept that the proteins are recognising particular distortions in the shape of the RNA molecule, rather than recognising specific nucleotide sequences common to all the polysomal RNA species of that cell. This follows simply because the evidence to date does not suggest that different eukaryote mRNAs have as many as four to seven specific nucleotide sequences in common. However the local nucleotide sequence in any region of high secondary structure could still determine the final degree of binding, so that dissociation constants of particular RNA-protein complexes could vary widely.

DO mRNA-ASSOCIATED PROTEINS BIND TO SEQUENCES COMMON TO MANY mRNA MOLECULES?

With the evidence, previously described, that all of the polysomal mRNA species of a cell are associated with the same discrete set of

tightly bound proteins, the possibility was raised that such proteins were recognising nucleotide sequences common to all eukaryote mRNAs (Blobel, 1973). It is widely established that poly(A) segments are a characteristic of all animal cell mRNAs, with the exception of histone mRNAs. Such poly(A) regions could provide binding sites for some of the mRNP proteins. Kwan and Brawerman (1972) were able to isolate a structure containing poly(A) complexed with a pronase sensitive particle from mouse sercoma polysomes. The poly(A) in the complex remained available for interaction with poly(U). [In addition the isolation of the mRNA-protein complexes by affinity chromatography to oligo (dT) cellulose reported in this investigation and in Lindberg and Sundquist (1974), suggested that at least a portion of the poly(A) sequences of animal mRNPs are available for base pairing with oligo (dT), i.e. are uncovered.] Kwan and Brawerman (1972) also claim that their unpublished results show similar poly(A) containing structures could be isolated from the non-polysomal 'free' mRNP.

The mRNP complexes released from the polysomes of mouse L cells and rat hepatocytes by puromycin were shown to contain two major proteins similar in molecular weight to the two proteins found associated with rabbit globin mRNA (Blobel, 1973). Polysomes from L cells were digested with RNAse after dissociation by puromycin and the digestion products analysed on sucrose gradients. A protein of molecular weight 78,000 could be detected in three separate fractions covering the sedimentation range 4-14s. Sucrose gradient analysis of [^{3}H] adenosine labelled polysomes revealed a peak of radioactivity at 11s, with a base composition of 80% AMP. Thus a poly(A) containing segment of mRNA cosedimented with a polypeptide of molecular weight 78,000. The sedimentation rate of the protein and of the RNA could be lowered by dissociating

the material released by the RNAse with either SDS or 2M LiCl. In summary mild nucleolytic treatment led to a release of mRNA segments associated with distinct proteins. Whilst the largest protein appeared to be bound to the poly(A) region of the mRNA, the binding site of the second largest protein (molecular weight 52,000) could not be elucidated. The author invoked the possibility that non-coding regions, possibly at the 5' end of the L cell mRNA, might provide a binding site for the smaller protein.

Do eukaryotic mRNAs then have zones of common base sequences other than adenylate rich regions at the 3' terminus? Little is known at the moment about the possibility of common non-coding sites (other than poly(A) sequences) existing within the mRNA species of a particular cell, but recent evidence indicates that speculation on this point is, at least justified.

Polysomal mRNAs from developing Xenopus embryos tested in an excess DNA hybridisation system appeared to contain two main components renaturing at different rates (Dina, Crippa and Baccar, 1973). The authors interpreted their results as suggesting that the fast reassociating region was transcribed from a family of homogeneously repeated sequences, and this small stretch was covalently attached to the main part of the RNA molecule transcribed from unique DNA sequences. Such identical repeated sequences attached to many unique RNA sequences would solve the problem of how a limited number of proteins can bind to all the polysomal mRNA species of a cell type. A preliminary report has also appeared that claims that half of the DNA sites that hybridise with haemoglobin mRNA can be occupied by mRNAs from other sources, but not by ribosomal RNA (Saloma et al. 1974). The authors believe that eukaryote mRNAs may contain

common base sequences. Such base sequences could correspond to repetitive areas of the DNA, but such sites may of course vary from species to species.

Whilst these findings are intriguing, they await confirmation. In particular it must be confirmed that the repeated sequences are covalently attached to the unique sequences. It is possible that two classes of mRNA exist, one transcribed from unique sequences, the other from repeated sequences, and that hybridisation of a mixed population of these two types would explain the renaturation rates of the Xenopus experiments (Campo and Bishop, 1974).

One might expect that the initiation region of related mRNA species may have some feature in common which could provide a specific binding site for the mRNA associated proteins. However polypeptides similar or identical to those associated with the mRNA of normally growing KB cells can also bind to adenovirus mRNA (Lindberg and Sundquist, 1974). It is much more difficult to envisage that viral and eukaryote mRNAs could have a number of nucleotide sequences in common, that could bind specifically the same protein(s), but it may be significant that poly(A) sequences have been found in the nuclear and polysomal RNA of adenovirus (Philipson et al. 1971).

Clearly further studies are required to clarify the nature and position of the binding of the proteins to RNA molecules. At the moment, the view that such proteins could be bound to sequences held in common by a wide variety of mRNA species appears at least tenable. Speculation on this concept was initiated mainly by the finding that a polypeptide of molecular weight 78,000 was bound to the polyadenylate region of a number of mammalian RNAs (Blobel, 1973). It has not been shown however that this protein is confined to the polyadenylate sequences. The

protein also cosediments with RNA fractions that do not contain a substantial amount of AMP. Considerable amounts of this protein can also be found associated with the small ribosomal sub-unit. This might reflect, as the author suggests, incomplete dissociation of mRNP from the small ribosomal sub-unit. Alternatively the protein might also be attached to regions other than the poly(A) sequence, and involved in the interaction of the mRNA with the ribosomal sub-units.

Other observations also indicate that it is unlikely that this polypeptide is confined to the poly(A) sequence. When mRNP complexes (isolated by affinity chromatography on oligo(dT) cellulose from KB cell polysomes) were prepared at high salt concentration, a component of molecular weight 78,000 was the main polypeptide remaining associated with the mRNA (Lindberg and Sundquist, 1974). However the mRNP particles released by EDTA treatment of high salt washed polysomes, ~~bound~~ are found in a narrow density range in CsCl gradients, indicating that the protein:RNA ratio of 0.7 between differently sized mRNAs did not vary greatly (Kumar and Lindberg, 1972). To maintain the same protein:RNA ratio over the whole size distribution of RNA from these mRNP fractions would require 2-20 molecules of the 78,000 molecular weight component per mRNA (Lindberg and Sundquist, 1974). Since the size of the polyadenylate region will not vary considerably between mRNAs of different size, it appears highly likely that the bulk of the protein molecules will be bound to additional sites on the mRNA. Even if the protein complement was made up by additional polypeptides, thereby decreasing the number of molecules required of any particular species of polypeptide, the number of binding sites associated with an average sized mRNA would be considerable.

SUMMARY

Where indications are available of the number of protein molecules bound per mRNA, namely the considerations of Lindberg and Sundquist (1974) outlined above, and the electron microscopic pictures of duck globin, (Dubochet et al. 1973), the data suggests that a considerable number of protein molecules are associated with each mRNA molecule. Taken together with the evidence presented in this report and in Brian and Hayashi (1973), that a limited set of proteins are bound to all the polysomal mRNA species of a particular cell type, one must explain a multiplicity of binding sites per mRNA sufficiently similar on different templates that a common set of proteins can bind to all. This binding seems unlikely to be due to specific nucleotide sequences in the mRNA, i.e. to sites held in common by different mRNAs, unless the degree to which specific mRNAs have sequences of bases in common has been underestimated to date by DNA-RNA hybridisation studies. Consequently the data at present suggest that the protein may be distributed along the mRNA molecule, the site of binding determined, in the main, by some factor other than specific nucleotide sequence. The proteins may recognise, initially, regions of high secondary structure, as suggested by electron microscope studies (Dubochet et al., 1973). A simple scheme to determine the specificity of the binding of those proteins to various mRNAs undergoing translation is outlined in the next section. In addition a method is suggested for determining whether specific polypeptides are confined to the polyadenylate region of mRNA molecules. Even if a considerable amount of information emerges from these sort of studies, it is clear that even more elaborate techniques will be required to elucidate the exact mode and site of binding.

Although several roles for the protein of mRNP particles have been suggested, such as stabilisation of mRNA and protection against nuclease activity, or changing the rate or efficiency of translation, the function of the proteins remains unknown (review Williamson, 1973). However, where attempts have been made to determine the biological significance of the mRNA-associated proteins, the evidence has implicated the proteins with involvement in the very initial stages of polypeptide synthesis.

The first evidence of a physiological role for the proteins in mRNP complexes came with the discovery that 14s rabbit globin mRNP could bind to native 40s ribosomal sub-units washed with sodium deoxycholate, whilst deproteinised 9s mRNA exhibited no such binding. (Lebleu et al. 1971). The authors reported that the binding of naked 9s mRNA to unwashed 40s ribosomal sub-units occurred to a significant extent, but other workers have found no difference in the binding of globin mRNP and mRNA to such sub-units (Pragnell and Arnstein, 1970). It is possible however that the proteins responsible for the binding remain associated with crude unwashed ribosomal preparations, thus permitting the mRNA to bind. The inability of the 9s mRNA to bind to the deoxycholate treated 40s ribosomal sub-unit remains surprising. Where additional cytoplasmic factors are employed, globin synthesis can be achieved. Thus liver sap has been used to translate globin mRNA on salt washed ribosomes (Sampson and Borghetti, 1972).

Other results indicating that the mRNA-associated proteins are of biological significance, implied a role in the formation of initiation complexes with natural mRNAs (Ilan and Ilan, 1973). Initiation factors 1_1 and 1_2 were prepared from a high salt wash of Tenebrio pupal ribosomes containing no endogenous mRNA. In a cell free system, these factors

translated the synthetic mRNA AUG$(U)_n$ but not natural mRNA. When the protein from 16s mRNP was separated by high salt treatment and chromatography and added to the cell free system, both natural and synthetic mRNAs were translated. In addition ribosomal wash from ribosomes possessing mRNA also initiated polypeptide synthesis under the direction of both synthetic and natural mRNAs. The factor separated from the 16s mRNA-protein complex (designated I_3) whilst required for the initiation of natural mRNA translation, had no capacity on its own to initiate polypeptide synthesis, whilst I_1 and I_2 individually, had very limited capacities. The results indicated that a protein factor bound to mRNA was necessary for the translation of natural message, and the authors suggested that the protein recognised a non-coding nucleotide sequence that preceded the AUG initiator region of mRNA. The factors involved in the binding and recognition of such regions are the subject of much research work at the moment, and recent results allow one to suggest and evaluate possible roles of the mRNA-associated proteins with much more confidence than hitherto. However before this evidence is considered, it should be pointed out that mRNA-bound proteins can be clearly separated from initiation factors by a high salt wash (Nudel et al. 1973). Furthermore these authors showed that the most mature rabbit reticulocytes no longer have active initiation factors in the 0.5M KCl ribosomal wash, but they do retain a normal amount of 14s mRNP. This persistence may reflect a basic role in the initiation process.

The results of several recent investigations suggest that the secondary structure of viral mRNA molecules may confer specificity in polypeptide chain initiation. The specificity directed by R17 RNA in an <u>in vitro</u> system with <u>E. coli</u> ribosomes is drastically altered by fragmentation of the RNA molecule (Steitz, 1973). Normally the intact

genome directs the synthesis of 20 molecule of coat protein and 5 mol. of replicase for every mol. of A (maturation) protein. Ribosome binding site preparations containing a mixture of the initiator regions of the three genes were rebound to various ribosome extracts, and their ratios in the 70s peak assessed. Surprisingly E. coli ribosomes did not discriminate in favour of the coat site, but preferentially bound the A-protein initiator fragment. The A-protein initiator fragment was recognised selectively some 40 fold and 11 fold over the coat and replicase sites respectively. Ribosomes from Bacillus stearothermophilus normally initiate only the A protein when directed by intact R17 RNA. When incubated with mixtures of the three initiator sites, the A protein initiator region was specifically rebound even where the R17 coat site was present in 10-fold molar excess. The discrimination ratio for the A site relative to the coat and replicase initiators was 40:1 and 8:1 respectively. Confirmation that some feature of the A-protein cistron ensures a high affinity for ribosomes was obtained by experiments with randomly fragmented R17 RNA. As the length of the degraded genome shortened, the relative initiations of the coat and replicase sites diminished remarkably. In contrast when the A site was liberated from the rest of the messenger molecule the A protein initiator region showed a steady increase in activity.

The authors believe that the ability of the R17 coat and replicase sites to be efficiently recognised may require the participation of regions of the phage genome beyond these binding sites. Their basic thesis postulates that the overall secondary and tertiary structure of the messenger molecule is important for ribosome recognition of initiator regions, and the activity of a particular initiator region can be enhanced or decreased by the remainder of the mRNA molecule.

Even if there were no feature within the immediate vicinity of the initiator codon that would promote a high affinity for ribosomes, other portions of the molecule could be arranged so that the initiator region remains in a highly prominent position, accessible for ribosome binding. Enhancement of activity could also occur if the rest of the RNA molecule was facilitating the opening of local hydrogen-bonded loops during ribosome attachment. Alternatively the secondary structure of the intact molecule may lead to the sequestering of the initiator codon. This could also occur if cistrons were hydrogen bonded to segments of adjacent cistrons, thereby decreasing initiation rates.

Thus it appears possible that different types of initiation sites occur, whose efficiency is innately determined by the secondary structure of the messenger molecule. It is tempting to speculate that cellular factors have evolved which recognise initiator regions and selectively expose the initiator codons so that the innate restrictions of secondary structure are overcome.

Direct physical evidence for the base-paired secondary structure of a fragment of mRNA in solution has been obtained. Both resolution of the time dependence of the melting curve for the RNA fragment (Gralla et al. 1974) and nuclear magnetic resonance studies (Hilbers et al. 1974) have established that the RNA is highly base-paired.

Evidence supporting the existence of non-random base-pairing also exists for bacteriophage T_4 lysozyme mRNA (Ricard and Salser, 1974). Following their studies on the size and folding of this messenger, the authors explain their results by a model in which mRNA secondary structure controls initiation at ribosomal attachment points. Lysozyme mRNA activity extracted from T_4 infected *E. coli* cells sedimented in several discrete peaks. Treatment with T_1 RNAse inactivated the lysozyme mRNA although the sedimentation profile remained similar to the undigested

control sample. Corresponding peaks were still found in sedimentation studies employing denaturing conditions. However when the mRNA was heat disaggregated briefly at 80°C, then treated with nuclease, the lysozyme mRNA activity was found in smaller fragments, furthermore some of the lysozyme mRNA activity was recovered which was initially lost by the nuclease treatment. Heat treated and nuclease digested samples showed a considerable increase in mRNA activity when subjected to a second heat disaggregation step.

The authors argue that if the secondary structure of the mRNA is highly ordered, many of the single strand breaks induced by the T_1 nuclease may remain masked. The lysozyme mRNA molecule could sediment as a single unit because the cleaved fragments were still held together by base-pairing. The second disaggregation step then revealed these specific breaks, allowing the lysozyme mRNA activity to be recovered in a number of smaller fragments.

To explain how nuclease treated lysozyme mRNA recovers its activity through denaturation by heat, the authors invoke the idea that secondary structure of the mRNA molecule may prevent translation of the lysozyme template. In their model the lysozyme messengers contain at least two ribosomal attachment sites. Ribosomes attach at one exposed site and continued translation into the lysozyme cistron. The second site remains base-paired, thus explaining how nuclease treatment inactivates the lysozyme mRNA. However this base-pairing is broken down by the second heat disaggregation procedure, which releases a small active mRNA fragment containing the now exposed second ribosome attachment site. Possibly the lysozyme mRNA is always produced in an inactive, highly base-paired form. Conformational changes in the mRNA molecular structure or specific nuclease cleavage could then bring about exposure of the ribosome binding sites at some particular stage of infection.

Both investigations discussed above emphasise the role of mRNA secondary structure in the control of initiation of protein synthesis. This secondary structure is in fact an intrinsic property of all mRNAs. Random nucleotide sequences generated by computer were evaluated according to the rules governing thermodyanic stability and most structures showed about 40-60% base-pairing (Gralla and De Lisi, 1974). Statistical analysis indicated that the probability of finding a t-RNA sized sequence, either random or real, that did not show secondary structure was very small. Thus the degree of base-pairing found in virus mRNA (on average about 60%) cannot be dismissed as a special adaptation for, say, virus packaging. Rather the extent of base-pairing exhibited by random structures imply that a high degree of secondary structure is a feature of all mRNAs.

The interaction between the bacterial initiation factor IF_3 and RNA has been studied in order to elucidate the relationship between particular secondary structures, such as hairpin loops, and the recognition of initiator region of the mRNA molecule (Wickstrom, 1974). The author employed nitrocellulose filter assays and equilibrium dialysis measurements to determine the degree of binding of the initiation factor IF_3 to various RNAs containing hairpin loops and single strand regions. For oligmers of less than 20 nucleotides, IF_3 bound with hairpin loops and single strands provided they contained the nucleotide sequence AUG. The RNAs lacking AUG did not bind IF_3. When longer single standed RNA such as a 70 base sequences of poly (A,U,G) was competed with a 300 base sequence of the single stranded poly (U), the poly (U) bound IF_3 equally well as poly (A,U,G). Thus IF_3 can bind nonspecifically to single stranded RNA, but as the author points out, with a high degree of base-pairing in natural mRNAs, such long single

stranded regions necessary for this type of binding will occur only rarely. In addition, the non-specific type of binding is composed of many weak interactions at many sites, whereas the specific affinity for AUG involves a strong binding per nucleotide. In summary, the binding of IF_3 is AUG specific for short oligmers but non-specific weak binding can occur with long single stranded regions. These are combined in the author's model for IF_3 behavious: the IF_3 factor binds first to the initiator codon within an exposed hairpin loop or internal loop, the base-paired region adjacent to this codon is then denatured, so that the mRNA initiation region is maintained in a single stranded state to facilitate binding to the small ribosomal sub-unit.

This model in which initiation sites previously rendered inaccessible by hydrogen bonded loops, become available for ribosome binding, is the first indication of how cellular components contribute to recognition of initiator regions. Whilst the initiation factor presumably denatures the hydrogen bonded loop with the most accessible initiation codon, and the weakest base-pairing, this ability to denature particular initiator regions may vary from one IF_3 factor to another, so that some IF_3 factors may exhibit mRNA specificity.

As pointed out earlier the two major observations on the functions of the proteins associated with eukaryote mRNAs both implicate these components in some aspect of the initiation of protein synthesis. With the insight provided by viral studies on the role of mRNA secondary structure and of cellular factors in the recognition and availability of initiation regions of mRNA, it appears reasible that the mRNP proteins are involved in the recognition of initiator regions.

Direct support for this contention comes from studies on two initiation factors involved in the formation of an initiation complex

with rabbit reticulocyte ribosomes (Cashion and Stanley, 1974). Both of these factors can be extracted from a .5M KCl polysomal wash. IF_I forms a stable ternary complex with GTP and initiator t-RNA in the absence of ribosomal sub-units, and promotes binding of met-t-RNA to the small ribosomal sub-unit. These complexes can be released on Millipore filters. IF_{II} is isolated from IF_1 by sequential step elutions from DEAE-cellulose. This factor is required for the transfer of complex containing an 80s ribosome. After this IF_1 mediated transfer, the initiator t-RNA is bound to the 80s ribosome at the P site where the methionine residue of the t-RNA is in position to form the first peptide bond. That such an initiation complex has been generated can be tested by determining the reactivity of the methionine residue with puromycin. The binding of the initiator t-RNA to ribosomal sub-units and ribosomes was analysed by sedimenting the initiation complex assembly through 10-25% exponential sucrose gradients. IF_I promoted the binding of the initiator t-RNA to the 40s ribosomal sub-unit, a step which required GTP but which is template independent. However the IF_{II} mediated transfer of the initiator tRNA from the 40s ribosomal sub-unit to the initiation complex on the 80s ribosome was promoted greatly by certain templates but not others. The oligmer AUG or the single stranded polymer $AUG(U)_{25}$ greatly promoted the transfer with about the same degree of effectiveness. An initiation complex could also be formed in the presence of rabbit globin mRNP, although not with the same efficiency as the synthetic RNAs. Surprisingly, however, rabbit globin mRNA only contributed to a level of initiation complex formation to that found in the presence of IF_I and IF_{II} alone. It appeared that with natural messengers containing a high degree of secondary structure,

the mRNP proteins were required for the formation of the initiation complex. When the relative amounts of (^{35}S) methionyl-puromycin formed in the initiation complex were compared, the data indicated again that the rabbit globin mRNAs could not participate in the complex formation. The relative amount of methionyl puromycin formed was approximately equivalent to that observed where no exogenous template was present, only IF_I and IF_{II}. In contrast the relative amounts of methionyl-puromycin formed in the initiation complex promoted by rabbit-globin mRNP or AUG(U)25 were very similar, equivalent to about 80% of that promoted by AUG.

The authors propose that "the mRNA-associated proteins establish the proper reading frame on the natural mRNAs by making accessible to the ribosome the correct AUG codon to serve as the initiator codon." This idea is a very attractive one, but the evidence would be more compelling if the difference in behaviour between the rabbit globin mRNP and rabbit globin mRNA were more marked. The number of (^{35}S) methionine counts found in the initiation complex on the 80s ribosome is only about 3.4 times higher when promoted by mRNP rather than mRNA, including correction made for the different amounts of template used in the experiment. Similarly, compared to mRNA, mRNP directs about three times the relative amount of methionyl-puromycin formation, but the total amounts formed in these assays, are very low. Other authors using equivalent amounts of reactants in a similar bacterial assay have reported the formation of p.mol. amounts of initiator amino-acyl puromycin complexes up to 70 times greater than that found for the rabbit reticulocyte assay (Bernal et al. 1974). If these results were repeated on a scaled-up version of the experiments, however, the evidence would indicate, in the clearest fashion to date, the role of the mRNA-associated proteins.

If the mRNP proteins play a role in translation, one might expect that their effect on the efficiency of translation might be revealed by comparisons of the rate of protein synthesis directed by mRNP or protein free mRNA in cell-free systems. In fact conflicting evidence exists in the literature as to whether the proteins associated with polysomal mRNA enhance translation in heterologous cell-free systems. Early experiments with in vitro systems suggested that mRNP from rabbit reticulocytes stimulated cell-free protein synthesis, in contrast to deproteinised mRNA which had an inhibitory effect (Weisberger and Armentrout, 1966; Lockard and Lingrel, 1969). With the refinement of cell-free systems it appears that in rat liver at least, there are no pronounced differences between reticulocyte mRNP and naked reticulocyte mRNA either in stimulating new protein synthesis or in the inhibition of amino-acid incorporation at higher concentrations (Sampson et al. 1972). The most recent work on the comparison of the activity of rabbit globin mRNP with that of protein free globin mRNA has now employed an embryonic brain cell-free system (Hendricks et al. 1974). The use of this ectodermal tissue may provide for a more valid comparison, since rat liver is erthryoporetic in embryos and may possibly retain cellular factors that can enhance the translation of erthryoporetic messengers. The authors data suggested that at a concentration of 0.4 A_{260} units/ml each of globin mRNP and globin mRNA, the mRNP stimulated the incorporation of (^{3}H) leucine into protein at about a 20% higher rate. At lower concentrations this difference is much less marked. Similar results were recorded for ascites cells although here the degree of increased incorporation appears to be about 15-17%. If one takes into account that the protein in the mRNP contributes slightly to the absorbance at 260nm, the stimulation by mRNP, on an RNA basis, is even greater. In fact the authors claim that mRNP stimulated amino-

acid incorporation by the cell-free systems at a rate 30-40% higher than the deproteinised mRNA, although they do not give the basis for this calculation. What is clear is that the mRNP did consistently stimulate protein synthesis in the embryo brain systems more than the corresponding mRNA.

The conflicting *in vitro* results for the mRNA-associated protein mirrors the present controversy as to whether initiation factors in eukaryote cells are messenger specific. A recent review listing the evidence for and against this concept has appeared, and the conclusions reached appear equally applicable to mRNA-associated proteins (Pain and Clemens, 1973). The authors make two points, firstly that the factors may recognise rather large classes of mRNA, discriminating between these classes through differences in conformational states or secondary structure. Secondly, with *in vitro* systems only the *relative* efficiencies of translation for different messengers may be altered by specific factors, and when mRNA is present in large amounts this effect may not be detected.

In any comparison between the relative efficiencies of mRNP and protein-free mRNA in stimulating protein synthesis in cell-free systems, there is always the possibility that the cell sap contains, even in limited amounts, similar proteins capable of binding to the naked mRNA, which attach to the mRNA before initiation, so making the comparison invalid. This objection cannot be overcome by using *in vitro* systems, since such proteins capable of binding many introduced mRNAs are even more likely to be present. The crucial experiment in this field, to evaluate the importance of the mRNA-associated proteins has not yet been performed. This is to re-isolate a specific mRNA from a heterologous cell-free system and determine whether it has become

associated during translation with the same set of proteins as those shown to be tightly bound to mRNA by established methods (Williamson, 1973).

I hereby propose an experimental rationale to answer this question, and in addition, to determine whether specific polypeptides are confined to the polyadenylate region of mRNA molecules.

The major requirement is for highly labelled mRNA-associated proteins. Unlabelled proteins of this sort, obtained by EDTA or puromycin induced dissociation of ribosomes, or by affinity chromatography on oligo (dT) cellulose, should ideally be labelled to a very high activity with 131 iodine. The protein moiety of rabbit globin mRNP has been labelled with (^{14}C) iodacetic acid (Temmerman and Lebleu, 1969). It may also be feasible to label the cell source of the proteins with (^{32}P) phosphate, since several of the duck globin mRNA-associated proteins are known to do phosphorylated (Morel et al. 1973). This method may be preferable if excess iodination interfered with the biological activity of the proteins.

The specific messenger can then be translated in a cell-free system derived from the wheat germ embryo containing the highly labelled mRNA-associated proteins in solution. Such a system has the advantage of a very low rate of endogenous protein synthesis. At the end of the incubation period, say 30 minutes, high ionic conditions can be chosen which terminate the synthetic activity of the system, and at the same time reduce fortuitous associations between mRNA and protein. It might be feasible at this stage to remove ribosomes and ribosomal sub-units by centrifugation, although this step is not essential. Either the supernatant or the whole cell-free system can then be passed, under the usual conditions for

isolating mRNPs though an oligo (dT)-cellulose column (Lindberg and Sundquist, 1974). The nature of the RNA moieties and the protein components can be analysed separately in subsequent stages. An oligo (dT)-cellulose column made up in lower ionic conditions to isolate naked poly (A) containing RNA (Lindberg and Sundquist, 1974), would provide a control to determine the amount of non-protein bound mRNA. If the mRNA has become associated with proteins during translation, analysis of the radio-activity of the proteins will reveal whether or not these are the mRNA-associated proteins originally isolated and suspended in the cell-free system. If these proteins were found in such an experiment, to bind to the mRNA during translation, this would emphasise the importance of the proteins in some basic role of polypeptide synthesis, and form the basis of future experimentation aimed at elucidating the exact role of the mRNA-associated proteins. Clearly it would be possible to compare the binding of a set of proteins with mRNAs from very different species, giving possible insights as to how the proteins recognise the mRNA, as well as indications on the specificity of the binding.

In addition, if such binding could be faithfully monitored, it should be possible also to determine whether the proteins bind only to specific nucleotide sequences or in some more general fashion. This follows from the discovery that the poly(A) sequence is not necessary for the translation of mouse globin mRNA in a cell-free system (Humphries et al. 1974). It would be of great interest to determine whether the polypeptides associated with complete mRNA molecules were identical in composition and amount to those capable of attaching to de-adenylated mRNA. With such a protein binding assay, it should be possible to determine whether specific polypeptides are confined exclusively or not to the poly (A) region of the mRNA molecule. Evidence on this point

could clarify the nature of the protein binding, and provide the basis for determining eventually the stoichiometry of the mRNP complexes.

The scheme appears feasible. All of the techniques, with the exception of the labelling of the mRNA-associated proteins, are currently available to this laboratory, and the majority have been used in the investigations described here. The difficulty is in obtaining a sufficiently high degree of labelling so that minute amounts of protein can be analysed. Alternatively one could employ a mRNA of high specific activity, achieved again by iodination or (^{32}P) labelling, but the advantage of a source of labelled proteins is that a number of different mRNAs could be tested for their ability to bind these proteins within absolutely equivalent cell-free systems.

Recent speculation on the role of mRNA-associated proteins

The nature of the protein components in mRNP has attracted much interest, but the lack of any assigned function for these proteins makes their study difficult. The most recent postulations on the function of at least some of the mRNA-associated proteins involve their interaction with the poly(A) sequence of eukaryote mRNAs. The precise function of polyadenylic acid sequences at the 3-OH terminus of eurkaryote mRNAs is far from clear, although recent evidence at least rules out that this sequence is necessary for initiation, re-inititon, elongation, translation or termination of protein synthesis. Other results indicated that the poly(A) sequences may be involved in the determination of the lifetime of a mRNA in the cytoplasm. However even more recent results question this hypothesis. Clearly this whole area of work is in a state of flux.

Speculation that the poly(A) sequences were a prerequisite for translation seemed unlikely in view of the fact histone mRNAs apparently do not contain poly(A) sequences (Adesnik and Darnell, 1972). Direct evidence has now been obtained to show that poly(A) sequences are not obligatory for the successful translation of mouse globin mRNA (Williamson et al. 1974) nor rabbit globin mRNA (Sippel et al. 1974). Deadenylated mRNA prepared by incubation of polysomal mouse globin mRNA with the exonuclease polynucleotide phosphorylase made globin at only 48% of the control level, in a Krebs ascites cell-free system. This reduced efficiency may merely reflect the presence of contaminating endonucleases degrading other regions of the molecule. When rabbit globin mRNA was hybridised with poly(dT) and then incubated with the endonuclease RNAase H, the deadenylated mRNA had identical translation properties to the original adenylated controls (Sippel et al. 1974).

The deadenylated globin mRNA competed as efficiently as normal globin mRNA against ovalbumin mRNA in an *in vitro* protein synthesising system, reducing ovalbumin synthesis to a comparable extent. The concept that removal of poly(A) sequences from mRNA results in unchanged translational activity is supported by the demonstration of repeated initiation of protein synthesis by deadenylated mouse globin mRNA in a rabbit reticulocyte lysate system (Humphries et al. 1974).

The poly(A) sequences could conceivably be involved in the regulation of the lifetime of mRNA. When the residual activities of globin mRNAs were measured in a preincubated Krebs ascites cell system, supplemented with reticulocyte initiation factors, a difference in comparative stabilities of normal and deadenylated mRNA activity was observed (Sippel et al. 1974). Intact globin mRNA appeared more stable showing a delayed decay curve of convex shape, whereas the activity of deadenylated mRNA decreased linearly with no period of delay. The poly(A) segments of mRNAs in sarcoma cells become shorter with age (Mendecki et al. 1972), and in Hela cells this process is independent of protein synthesis (Sheiness and Darnell, 1973). Since molecules without poly(A) sequences such as histone messengers and bacterial messengers have very short half lives, there has been much speculation that the functional lifetime of enkaryote messengers may be determined by the rate at which the poly(A) segment is progressively shortened, to leave the sequences behind this region susceptible to nuclease attack. Interactions between the mRNA-associated proteins and the poly(A) sequence may then determine the rate at which the poly(A) sequence is degraded. Strong support for this type of contention comes from recent work on the comparitive rates of processive phosphorolysis of rabbit globin mRNA and mRNP, using a molar excess of polynucleotide phosphorylase (Soreq et al. 1974). Using this

enzyme at 0°C, in the presence of I m NaCL to retard the rate of phosphorolysis, the authors could remove the poly(A) sequence, apparently without degrading the rest of the molecule. Whilst the rate of phosphorolysis of the poly(A) sequence of the globin mRNA was identical to that of free poly(A), when the mRNA was contained in a polysomal ribonucleoprotein complex, the poly(A) sequence was protected from the action of the enzyme. Whilst these results suggest that the attachment of the mRNA-associated proteins extends to the 3-OH end of globin mRNA, their masking or protective effect could be overcome by using the enzyme at 37°C. The region of the mRNA molecule behind the poly(A) sequence appeared to have a stable secondary structure, since nucleoside diphosphates other than ADP were only slowly liberated by phosphorolysis. The authors also reported that the initial rate of translation of deadenylated mRNA, in a Krebs ascites cell-free extract, was virtually identical to that of intact poly(A) containing mRNA. However at longer periods of incubation, the rate of protein synthesis had a tendency to level off more quickly with the poly(A) free mRNA than with intact mRNA. The presence of the poly(A) sequence in the mRNA molecule is not necessary for its translation but its removal appears to shorten the time period during which the mRNA can code for protein synthesis in the cell-free system. These results agree well with those obtained by specifically removing the poly(A) sequences from rabbit globin mRNA with Ribonuclease H (Sippel et al. 1974) but it should be noted that the latter authors were able to compare the stabilities of normal and deadenylated globin mRNAs since their Krebs ascites cell-free system was active for several hours, in contrast to Soreq et al.(1974) whose similar system was only active for about 90 minutes.

Recently, to overcome the time limitation and the inefficiency of

translation found in the in vitro cell-free systems, the translation of intact and deadenylated mRNA in Xenopus oocytes has been compared (Huez et al. 1974). (In this system the translation of intact exogenous mRNA has been shown to proceed for several days with a high degree of efficiency (Gurdon et al. 1973).) After about seven hours of incubation the rate of haemoglobin synthesis in oocytes injected with intact globin mRNA was equivalent to that of endogenous protein synthesis (Huez et al. 1974). After twenty hours of incubation it represented 142% of endogenous protein synthesis. At the end of the experimental period (48 hours) the intact globin mRNA was still being efficiently translated. In contrast the poly(A) free mRNA showed a decrease in the rate of translation compared to that of endogenous messengers. By twenty hours of incubation the rate of haemoglobin synthesis was only 24% of that of endogenous protein synthesis. The authors estimated that the functional half life of poly(A) free mRNA in the Xenopus oocytes is only from 5-10 hours though intact globin mRNA has a much higher half life in the oocytes than in the reticulocytes. Again these results indicate that the presence of the poly(A) sequence stabilises the mRNA molecule during translation.

One other possible interpretation is that the recycling of poly(A) free mRNA on the ribosomes is simply less efficient that that of authentic mRNA. If the messenger associated proteins and the poly(A) sequence are involved in the recycling of mRNA on the ribosomes, then their removal would lead to an eventual blockage of the translation process. (Soreq et al. 1974, Huez et al. 1974). This is not necessarily an exclusive interpretation since it is possible that the factor, if any, responsible for recycling of the mRNA on ribosomes could also prevent mRNA degradation, obviously mRNA-associated proteins are ideal candidates for this dual role. However this role in translation seems less likely in view of the demon-

stration that even deadenylated mRNA programmes repeated initiation of protein synthesis in an in vitro cell-free system (Humphries et al. 1974).

On balance, these results indicate that the poly(A) sequences play a regulatory role in messenger stability rather than messenger translation. This postulation is questioned, however, by a surprising devolopement in this field of work, which indicates that messengers entirely lacking in poly(A) sequences co-exist with poly(A) containing messengers. Thus more than 40% of the non-histone mRNA species in blastula stage sea urchin embryoes lack poly(A) sequences (Nemer et al. 1974). A comparatively high proportion (30%) of non-adenylated mRNAs can also be found in Hela cells (Milcarek et al. 1974). Furthermore for both the mammalian and sea urchin cells, hybridisation studies indicate that the mRNAs containing and lacking poly(A) contain different nucleotide sequences, suggesting that they represent two genuine populations and that the mRNAs lacking poly(A) are not derived by partial degradation of poly(A)-containing mRNA. The poly(A) free mRNA in Hela cells shows the same decay kinectics as poly(A)-containing mRNA (Milcarek et al. 1974). If this work can be confirmed with well characterised mRNAs coding for specific products, then the question of what function the poly(A) sequences play in mRNA molecules will be reopened. It would be interesting to compare the stability of naturally occorring poly(A) free mRNA with poly(A) containing mRNA in the oocyte system, to redetermine the relationship between messenger lifetime and the presence of poly(A) nucleotide stretches.

One suggested function for the poly(A) sequences is that they may interact, through the mediation of the mRNP proteins, with subcellular structures, possibly to produce separate poly-ribosomal distributions of the two mRNA populations, those containing poly(A) and those lacking a poly(A) sequence. (Nemer et al. 1974). Strong evidence for this

contention comes from the demonstration that, in Hela cells, the mRNAs found on polyribosomes associated with membranes contain poly(A) sequences attached directly to the membrane (Milcarek and Penman, 1974). After nuclease treatment little RNA other than poly(A) appears to remain on the membranes and the authors believe that this direct association of the poly(A) sequence with the membranes may be mediated through the mRNA-complexing proteins. Control mixing experiments appear to rule out the non-specific sticking of poly(A) sequences to membranes although the basis of this special affinity for membranes is unknown.

If mRNAs can be sequestered and attached to discrete classes of membrane bound polyribosomes by virtue of their poly(A) sequences and associated proteins, then the recent demonstration that rat liver RNAase activity is not distributed equally amongst different polyribosomal populations (Arora and Robert, 1974) may be of considerable significance in any speculation on the nature of differential messenger stability within the same cell. Thus it seems reasonable to postulate that the final arbiter of messenger stability may be the local RNAase activity and that the distribution of messengers to various polyribosomal populations may be mediated through the poly(A) sequence and its associated proteins. It would appear profitable to compare quantitatively and qualitatively those proteins associated with poly-A containing mRNA with those complexing with poly(A) free messenger. Are mRNAs found associated with membrane bound polyribosomes complexed with an identical set of proteins to those mRNAs found on free polyribosomes?

Other recent evidence shows several general lines of research converging, particularly on such intriguing questions as the relationship between mRNA turnover and poly (A) shortening, and poly (A)-mRNP complexes. Thus pulse chase studies, in addition to those described in the Introduction, have confirmed that cultured cell lines have major poly(A)-containing species

of mRNA that decay exponentially with a half-life of many hours (Murphy and Attardi, 1973, Cowan and Milstein, 1974). However a newly developed technique that involves pulse-labelling with guanosine detected mRNA of shorter half-life than that found with the more conventional uridine pulse chase techniques (Puckett et al., 1975). Using this technique, Sheiness et al., (1975) found that when HeLa cells were making protein normally, some 38% of poly(A) containing mRNA turned over within 2 hours of chase with unlabelled guanosine and actinomycin (5 µg/ml). In the presence of emetine, which inhibits protein synthesis by blocking ribosomal movement, the decay of mRNA was only half as fast. Furthermore this drug substantially diminshed the poly(A) shortening process. Thus both the apparent turnover and shortening of poly(A) lengths are retarded by slowing protein synthesis. The authors results indicate also that after long term labelling conditions a high proportion of molecules contain short poly(A) strands of less than 50 nucleotides, from an original stretch of poly(A) of about 200 nucleotides. As described in the Introduction, the bulk of recent work indicates that mRNA decays exponentially, i.e. messengers of all ages are equally likely to be lost. Molecules of all ages are replaced only by new messengers (with long poly(A) stretches), Sheiness et al., 1975. The authors suggest that random endonucleoytic clearage of poly(A) is the most likely method of accumulating short poly (A) segments rather than a variable exonucleolytic clearage which slows down as the segments shorten. Such a model can explain the random nature of mRNA decay, if endonucleolytic attack leads to scission of poly(A) to a size below which the mRNA is not stable.

It has also been reported that mRNP proteins can bind Met-$tRNA_f$ (initiator tRNA) (Hellerman and Shafritz, 1975). Some of the smaller polypeptides of reticulocyte mRNPs appear to be components of a specific eukaryotic initiator factor, the soluble Met-$tRNA_f$ binding protein.

Like this factor, mRNPs also bind poly(A) sequences. Poly(A) is bound to a much greater extent than poly(U) or poly(C) but other poly(A) binding proteins isolated from the cytoplasm do not bind initiator tRNA. (As pointed out above, mRNP proteins have been consistently implicated in the very early events of eukaryotic initiation, although no specific function has yet been described). The authors also report that supernatant mRNPs show the ability to bind Met-$tRNA_f$. The ability of the mRNP proteins to also bind poly(A) suggests that this sequence may act as a recognition site for protein binding onto the mRNA. Rigorous size constraints likely on such specific proteins as initiation factors may, in part, explain the similarity of patterns of mRNP proteins revealed by SDS-polyacrylamide electrophoresis. Isoelectric focusing, which measures the overall charge ratio of polypeptides, revealed differences in chick lens and rabbit reticulocyte mRNP patterns (Chapter 13). Investigations are continuing in this laboratory to determine whether this is a species or tissue difference. However proteins bound to nuclear heterogeneous RNA, appear to be much more complex than mRNP proteins, and species-species differences have been found (Pederson, 1974).

Note: It should be possible technically to evaluate directly the relationship of poly(A) shortening to mRNA in the lens system. The isolation at a specific age, of the stable and unstable mRNA should be possible using the immunological technique of Clayton and Truman (1970). Suitable candidates appear to be alpha-2 mRNA (stable) and delta-5 (unstable) - see Chapter 14. Exact sizing of the mRNAs and estimation of poly(A) lengths using the techniques described in Sheiness et al., (1975) should indicate whether mRNA stability is related to the shortening of poly(A) sequences or not. (The comparative data obtained in Chapter 14 assumes that any effect actinomycin has on aspects of protein synthesis, such as initiation events (singer and Penman 1973; Craig, 1973) apply to all mRNAs, rather than affecting mRNA-specific factors. This is a much less likely possibility but one that must be borne in mind).

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